AVERSION AND REWARD: TWO OPPOSING DRIVES MEDIATING ALCOHOL-SEEKING BEHAVIOR

by

Andrew Kraft Haack

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STATEMENT OF THESIS APPROVAL

The thesis of Andrew Kraft Haack has been approved by the following supervisory committee members:

Sharif Taha	, Chair	5/15/2015 Date Approved
Kristen Keefe	, Member	5/15/2015 Date Approved
Karen Wilcox	, Member	5/15/2015 Date Approved
Alan Dale Dorval II	, Member	5/18/2015 Date Approved
Christopher Gregg	, Member	5/15/2015 Date Approved

and by Richard Dorsky , Chair of the Interdepartmental program in Neuroscience and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Alcohol use disorders (AUDs) have had a devastating impact on the health and lives of those who struggle with AUDs. Given the impact of AUDs on human health, the neural mechanisms that underlie ethanol-seeking behaviors are a topic of intense interest. In this dissertation, I investigated the neural mechanisms of ethanol-seeking. Motivation for seeking ethanol can be characterized by learning about both the rewarding and aversive properties of ethanol. Indeed, increased drinking would be observed if an animal was more motivated for ethanol's rewarding effects. Furthermore, similar increases in ethanol consumption would be accompanied by an attenuation in learning about ethanol's aversive effects. In this dissertation, I explore the neural mechanisms of reward and aversion that may contribute to increased ethanol-seeking behavior.

First, to investigate how mechanisms of reward contribute to ethanol-seeking, I implemented the techniques necessary to record phasic dopamine (DA) signaling during ethanol-seeking (Chapter 2). Phasic dopamine release is distinguished by the subsecond release and reuptake of the neurotransmitter. Recent work has found that phasic DA release in the nucleus accumbens may be linked to motivation and performance of reward-seeking behavior but few studies have investigated phasic dopamine release during operant ethanol self-administration. In Chapter 3, I describe phasic DA release being evoked by cues

predictive of ethanol availability. Furthermore, the magnitude of the DA was found to be predictive of shorter lever press latencies. Finally, phasic DA release was evoked during the performance of an ethanol-rewarded lever press. The dopamine recorded during ethanol-seeking is consistent with the hypothesis that dopamine may mediate both the motivation for ethanol and performance of ethanol-seeking behavior.

Second, to investigate the mechanisms of aversive learning about ethanol, I focused on a brain region long associated with aversion, the lateral habenula (LHb). Recent work identified the LHb in learning about aversion to cocaine, thereby suggesting it may have a role in learning about the aversive effects of ethanol. In Chapter 4, I describe that lesions to the LHb caused an increase in home cage ethanol consumption. Furthermore, when an aversive injection of ethanol was paired to a novel tastant, the consumption of the novel tastant improved more rapidly than in intact animals. The above results implicate the LHb in learning about ethanol's aversive effects. Future studies must be performed to establish which LHb afferents mediate aversion to ethanol.

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CHAPTER 1

INTRODUCTION

Alcoholism is a grave societal and health burden

Alcoholism is a disease that can devastate the lives of alcoholics and the lives of their families and loved ones, all while imposing tremendous economic and health burdens at a societal level. A recent meta-analysis of the cost of alcoholism found that it incurs a cost of 1% of the total global gross domestic product by causing myriad health problems, lost productivity and costs associated with litigating and punishing alcohol-related offenses (Rehm et al., 2009). A recent analysis of data from the United Kingdom compared the harms of different drugs of abuse to both the user and those around the user and found that ethanol was the most harmful when compared to other drugs of abuse including heroin, cocaine, tobacco and methamphetamine (Nutt et al., 2010). Compounding the harm and cost associated with alcoholism is the relative lack of therapeutics effective in treating alcoholism. Only three medications are FDA approved for use in the treatment of alcoholism in the U.S., those three drugs have limited efficacy (Kranzler and Van Kirk, 2001), and what efficacy these drugs have is hindered by poor patient compliance (Volpicelli et al., 1997). The widespread detrimental effects of alcoholism highlight the importance of developing effective treatments to

alleviate the harm and substantial cost incurred by alcohol use disorder (AUD). Developing a more thorough understanding of the neural mechanisms underlying addiction to ethanol is an important first step in developing new medications to treat the disease.

Two competing learning processes during drinking condition future intake

Ethanol consumption has dose-dependent rewarding and aversive effects; both importantly contribute to regulating voluntary ethanol intake. These effects occur both during and after ethanol consumption to influence future consumption (Gilpin and Koob, 2008). The rewarding effects of ethanol occur relatively rapidly, and are thought to be associated with the ascending limb of the blood ethanol concentration curve (Lewis and June, 1990). Aversive effects, including motor incoordination, sedation and nausea, generally occur at longer latency and after ingestion of higher alcohol doses (Schramm-Sapyta et al., 2010). Aversive hangover effects can occur after drinking and often occur at even longer latencies from when initial aversion is felt by the user (Schulteis and Liu, 2006). Given that both aversion and reward can potently affect future behavior, these rewarding and aversive properties of ethanol may thus underlie the development of AUD (Schultz, 2006; Gilpin and Koob, 2008).

Rewarding effects of ethanol contribute to drinking behavior

Several lines of evidence using animal models have demonstrated that ethanol has rewarding properties, and these are likely to come from ethanol's interaction with the brain. The most commonly used method to demonstrate rewarding effects of a drug is operant self-administration. Here an animal must perform an action, such as a lever press, to receive a dose of the drug. Some of the first work to establish that ethanol has rewarding properties observed that rats will lever press for stomach infusion of ethanol, demonstrating that ethanol's reward is not related to its gustatory sensory properties (Waller et al., 1984). Furthermore, authors have made a direct link between ethanol's action on the brain and its rewarding properties, by demonstrating that rats will lever press to receive ethanol infusions directly into a specific region of the brain, the ventral tegmental area (VTA; Gatto et al., 1994; Rodd-Henricks et al., 2000). In addition to operant models, the conditioned place preference paradigm has been extensively used to demonstrate that mice prefer an environment that has been paired with ethanol exposure (Reid et al., 1985; Bozarth, 1990; Cunningham et al., 1991). These data firmly establish the rewarding properties of ethanol, and implicate actions of the VTA in ethanol's rewarding effects.

To further determine if a drug influences the functioning of the brain's reward systems, researchers use intracranial self-stimulation (ICSS). In this paradigm rats are trained to lever press for progressively lower intensities of brain stimulation until a minimum stimulation intensity capable of maintaining the behavior is reached. Many drugs of abuse attenuate the intensity of ICSS required to maintain

the lever-pressing behavior, suggesting the drugs potentiate the function of the brain's reward circuitry (Esposito and Kornetsky, 1977). Ethanol self-administration also decreases the level of stimulation required to maintain the lever-pressing behavior (Moolten and Kornetsky, 1990), suggesting that it too potentiates the brain's reward circuitry. Given the potent effect of ethanol on the reward circuits of the brain, these pharmacological effects of ethanol during self-administration may influence future ethanol-seeking behavior. In Chapters 2 and 3 of this dissertation, I investigate the role of the brain's reward systems during ethanol self-administration to elucidate how these systems may motivate ethanol seeking.

Decreased aversion to alcohol contributes to increased alcohol-seeking

Learning about the aversive properties of a substance has long been known to have a robust effect on intake. Indeed, it has long been known that pairing saccharin with gamma irradiation will substantially attenuate future saccharin drinking (Garcia et al., 1955). Following from this work, drugs of abuse have been proposed to have an aversive component and this aversion becomes more pronounced at higher doses of drug (Goudie, 1979). Ethanol exerts aversion through several mechanisms. While its pharmacology can induce aversion given a sufficiently large injection, it is most commonly consumed orally. The gustatory reaction to ethanol has also been found to induce aversion and may also limit intake of the drug. Given this evidence, it seems likely that an attenuated aversive response to both the pharmacological and gustatory sensation of ethanol may

have a powerful effect on ethanol-seeking behavior.

Two influential models of the development of drinking behavior suggest that an individual's initial sensitivity to the aversive effects of ethanol is predictive of their chance of being diagnosed with an AUD. Specifically, the low responding (LR) model posits that an initial low sensitivity to ethanol's aversive, as well as its rewarding, effects are predictive of the development of AUD (Schuckit, 1994). Because of their low sensitivity to the rewarding effects of ethanol, LR persons in their early drinking experiences are thought to consume more, in order to reach a level of ethanol consumption where the rewarding effects can be felt. Low sensitivity to ethanol's aversive effects in LR individuals thereby limits the aversive learning from these drinking experiences, leading to increased chance of developing AUD. In the differentiator model, low sensitivity to the aversive properties of ethanol and greater sensitivity to its rewarding effects are theorized to increase the risk of the development of AUD (Newlin and Thomson, 1990). A recent study provided evidence in support of this model (King et al., 2014). Recording responses to rewarding and aversive effects of ethanol from a group of human subjects and following these subjects for a 6-year period, the study found that ratings of potentiated reward and attenuated aversion to ethanol were predictive of the development of AUD. A commonality in both the LR and differentiator models is the prediction that attenuated reaction to the aversive effects of ethanol will potentiate future drinking. Taken together, these models suggest that learning about the aversive effects of ethanol and how these are encoded in the brain is critical to fully understanding drinking behavior.

Experiments in animal models suggest a similar relationship between sensitivity to the aversive effects of ethanol and voluntary ethanol intake; that is, *in*sensitivity to ethanol's aversive effects is associated with greater intake levels. For example, conditioned taste aversion (CTA) is a simple behavioral paradigm that has been widely used to measure the aversive effects of drugs of abuse. In a CTA experiment, a dose of the drug is typically administered noncontingently immediately after consumption of a novel and appealing tastant (often a saccharin solution). The novel tastant becomes associated with the aversive effects of the drug that occur after injection. The amount of aversion can be quantified by measuring the reduction in animal's voluntary consumption of the tastant in subsequent presentations. A number of studies of ethanol-induced CTA provide convergent evidence that sensitivity to the aversive effects of ethanol is accompanied by low levels of voluntary intake. Sardinian alcohol-preferring rats that have been bred to avidly consume ethanol experience attenuated CTA compared to Sardinian alcohol-nonpreferring rats (Brunetti et al., 2002). Furthermore, rats that show less ethanol-induced CTA during adolescence voluntarily consume more ethanol after an ethanol-deprivation period (Schramm-Sapyta et al., 2010). In addition, studies of inbred mouse lines have found that mice that experience attenuated ethanol-induced CTA display potentiated ethanol preference in the home cage and attenuated withdrawal severity (Broadbent et al., 2002). These data thus suggest that ethanol-induced aversion experienced by the drinker may be crucial in mediating subsequent drinking behavior. In Chapter 4 of this dissertation I present evidence demonstrating the involvement of a specific

brain region, the lateral habenula (LHb), in both learning about the aversive effects of ethanol and in regulating the rate at which escalation of voluntary ethanol intake occurs.

Learning about reward and aversion both contribute to alcohol-seeking

There is an important interplay between both reinforcing and aversive aspects of ethanol that lead to ethanol-seeking behaviors and AUDs (Gilpin and Koob, 2008). The signaling of the neurotransmitter dopamine (DA) in the nucleus accumbens (NAcc) has undergone extensive study for its crucial role in reward-seeking behavior (Wise, 2004; Bromberg-Martin et al., 2010a; Salamone and Correa, 2012). Following from this interest is work that has investigated the role of DA in ethanol-seeking (Gonzales et al., 2004). Yet, the published work is conflicting with respect to whether DA is involved in ethanol-seeking and what role it may play, if any.

Due to limitations of the technology used previously to record DA during ethanol-seeking (Schultz, 2007), the temporal dynamics of DA release during ethanol seeking is unclear. Furthermore, it is unclear how DA release may be related to specific ethanol-seeking behaviors. In this dissertation, I attempt to further our understanding of DA release during ethanol-seeking by applying the technique of fast-scan cyclic voltammetry (FSCV), a technique for recording extracellular DA levels with a high degree of temporal resolution.

One region that has been identified to encode both rewarding and aversive

stimuli is the habenular complex, in particular the LHb. While this region has long been hypothesized to have a role in behavior due to its afferent inputs (Herkenham and Nauta, 1977), only recently has it come under intense scrutiny to discern the nature of this role. Studies in nonhuman primates and rats have revealed a role for the habenula in responding to aversive stimuli (Rausch and Long, 1974; Matsumoto and Hikosaka, 2009). Further, work that has studied the role of the habenula during drug-seeking has suggested that it may be involved in learning about the aversive properties of these drugs (Fowler et al., 2011; Jhou et al., 2013; Meye et al., 2015). These findings suggest a general role of the habenular complex in mediating the influence of aversive outcomes in behavior, including the role of aversive properties of drugs of abuse in controlling drug-seeking behavior. However, a specific role for the habenular complex or the LHb in mediating ethanol-seeking behavior is presently unknown. In this dissertation (Chapter 4), I present evidence that strongly implicates the LHb in learning about the aversive properties of ethanol.

To examine the role of the habenular complex in ethanol-seeking and consumption, I explored the interplay between learning about the rewarding and aversive aspects of ethanol, and I have divided discussion of these investigations into three chapters. First, to allow the exploration of the role of DA in ethanol-seeking, I developed and implemented the FSCV and instrumental learning techniques necessary for recording DA release on a subsecond time scale during ethanol-seeking and consumption (Chapter 2). Second, I used these approaches to examine subsecond DA signaling in the NAcc during ethanol seeking and self-

administration (Chapter 3). Third, I examined the role of the LHb in learning about the aversive properties of ethanol (Chapter 4).

Developing technology to record DA during ethanol seeking

Ventral striatum in reward-seeking

The NAcc makes up a central part of the limbic loop of the basal ganglia. Here input from limbic areas such as the anterior cingulate and orbitofrontal cortices, along with the hippocampus and amygdala, funnel into the ventral striatum circuitry (Glees, 1944; Nauta, 1958; Russchen et al., 1985). The ventral striatum includes the NAcc and contains GABAergic cells called medium spiny neurons (MSNs). As the primary output cells of the striatum, MSNs combine inputs from a variety of afferents. In addition to excitatory glutamatergic input, the MSNs also receive neuromodulatory input from cholinergic interneurons and the DA afferents arising from the ventral tegmental area (VTA) (Fibiger, 1982; Swanson, 1982). The MSNs project to the ventral pallidum; from the pallidum projections continue on to the thalamus and cortex to influence behavioral selection (Redgrave et al., 1999, 2010). As the ventral striatum receives projections from a diverse array of brain regions, some have hypothesized that it serves as a crucial link between the limbic system and the control of motor output (Mogenson et al., 1980). Indeed, stimulation of limbic structures has long been known to lead to the generation of behavior (Hess and Akert, 1955). Given this hypothesis, the NAcc has come under intense interest for understanding the generation of reward- and drug-seeking behavior. Here, I focus on how DA signaling in the NAcc may be involved in

ethanol-seeking behavior.

Dopaminergic input to the ventral striatum

A significant portion of the brain's DA projections originate in two adjacent nuclei, the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNpc). The NAcc receives much of its DA input from the VTA, with input transitioning to originating from the SNpc in more dorsal and lateral regions of the striatum. The firing activity of DA neurons has been classified into two categories, referred to as tonic and phasic (Grace, 1991). Tonic activity consists of low frequency (1-5 Hz) rates of action potential firing (Grace and Bunney, 1983; Johnson and North, 1992; Nedergaard and Greenfield, 1992). Such low frequency firing is thought to result in rates of DA release resulting in extracellular concentrations of DA between 5-20 nM that change gradually on the timescale of minutes (Parsons and Justice, 1992; Hogan et al., 1994). This mode of firing is often referred to as arising from pacemaker activity of the DA neurons and is generated by channels mediating a voltage-dependent Ca^{2+} conductance in the SNpc DA neurons (Harris et al., 1989; Kang and Kitai, 1993; Nedergaard et al., 1993) and a voltage-independent Na^{+} conductance in the VTA (Khaliq and Bean, 2010).

Conversely, phasic activity is characterized by high frequency, synchronized bursts (14-30 Hz) of firing of large numbers of DA neurons (Grace and Bunney, 1984; Hyland et al., 2002). Burst firing of DADA neurons results in a transient increase in DA concentrations as high as 1 μM on a subsecond timescale

(Garris et al., 1997). The substantial and transient increase seen during phasic DA is thought to bind to different DA receptor subtypes than tonic DA, and therefore likely has differential effects on behavior (Gonon, 1988; Dreyer et al., 2010). Bursting activity and phasic DA release has been identified to depend on activation of NMDA receptors expressed on DADA neurons (Johnson et al., 1992; Zweifel et al., 2009). Furthermore, the synchronized pattern of firing is likely to require the combined activity of the laterodorsal tegmentum and the pedunculopontine tegmentum (Forster and Blaha, 2003; Lodge and Grace, 2006). This synchronized activity has been found to occur during the receipt of natural reinforcers, in addition to stimuli that are predictive of reward (Ljungberg et al., 1992; Schultz et al., 1997). Ablating this activity with genetic techniques has been found to lead to a disruption in learning about rewarding and aversive relationships (Zweifel et al., 2009). Given the apparent role of phasic, bursting activity on learning, accurate and reliable methods of recording DA are necessary.

Methodologies of recording dopaminergic signaling

Traditional methods of detecting the activity of DA neurons innervating the NAcc have used either *in vivo* microdialysis to measure extracellular DA levels in the NAcc or electrophysiological recordings of DA neurons in the VTA. *In vivo* microdialysis involves the use of cannulae that incorporate a semipermeable dialysis membrane at the tip, thereby creating a closed perfusion system that can be implanted into the anatomical region of interest (Westerink, 1995; Plock and Kloft, 2005). Artificial cerebrospinal fluid is perfused through this probe and, based

on size, compounds in the extracellular fluid surrounding the probe diffuse into the dialysis solution flowing within the probe and are collected. Dialysate samples are then analyzed "off line" to determine the nature and concentration of analytes. While very accurate and able to resolve a variety of neurotransmitters (Perry et al., 2009), dialysis is limited by its low temporal resolution (Watson et al., 2006). Sampling frequency of dialysis is on the order of minutes, thereby limiting the usefulness of this technique in detecting phasic DA release, particularly related to a specific behavior (Schultz, 2007). Electrophysiology, on the other hand, can detect the firing of action potentials with very high temporal resolution (Lewicki, 1998). However, ascertaining that one is actually recording from DA neurons has proved challenging (Ungless and Grace, 2012). First, the identification of DA neurons has been demonstrated based on electrophysiological characteristics of the neurons in the SNpc (Grace and Onn, 1989); however, recent evidence suggests that these markers may not be sufficient to identify DA neurons in the VTA (Margolis et al., 2006, 2008). Secondly, DA neurons of the VTA are known to project to a variety of locations (Swanson, 1982). This diverse set of projections therefore makes determination of the anatomical target of neurons during electrophysiological recordings difficult without antidromic stimulation techniques (Guyenet and Aghajanian, 1978). These weaknesses of the *in vivo* microdialysis and electrophysiology approaches have necessitated the development of a technique that can detect DA release in an anatomical region of interest with temporal resolution sufficient for identification of phasic release events.

Fast scan cyclic voltammetry

To satisfy these demands, the fast scan cyclic voltammetry (FSCV) technique was developed to detect phasic DA release in behaving animals (Phillips et al., 2003a). In this technique, a carbon fiber microelectrode is used to detect DA from the anatomical region of interest on a subsecond timescale. To detect DA, the implanted carbon fiber microelectrode is held at a potential of -0.4 V relative to an Ag/AgCl electrode implanted in contralateral forebrain. During a voltammetric scan, the potential applied to the carbon fiber microelectrode is ramped from -0.4 V to +1.3 V. The increasing voltage causes oxidation of DA into dopamine-o-quinone, and the resulting loss of electrons is detected at the electrode and recorded as current. Upon reaching +1.3 V the potential of the carbon fiber microelectrode is ramped back to -.4 V. The dopamine-o-quinone generated by the oxidation is then reduced back into by DA by the decreasing voltage. This negative voltage causes electrons to be gained from the electrode, which is recorded as negative current. The entire voltammetric scan is completed in 8.5 ms, and scans are repeated every 100 ms to achieve a sampling frequency of 10 Hz. Thus, the ability to record DA concentration every 100 ms provides the temporal resolution necessary for recording phasic DA release events on a behaviorally relevant timescale. Recent advances in FSCV have further refined the technique to allow chronic, long-term recordings and to reduce the size of the voltammetric implant (Clark et al., 2010). FSCV in its most recent form has become a flexible tool for the detection of rapid DA release *in vivo* during behavior.

Role of DA in mediating reward-seeking

The role of DA in reward-seeking was known long before the development of methods to detect DA in anatomical regions. Lesions of the majority of DA neurons produced a pronounced lack of reward-seeking behavior, including a complete lack of feeding (Zigmond and Stricker, 1972). This lack of reward-seeking after DA lesion applies to drug-seeking as well, as cocaine self-administration has shown to be attenuated after a DA lesion (Roberts and Koob, 1982). Furthermore, administration of drugs that antagonize DA receptors results in attenuated function of the brains reward systems, as determined by reduced ICSS behavior (Fouriezos et al., 1978). These findings led to the hedonic hypothesis of DA, where responding for rewarding stimuli is motivated by pleasurable effects that are mediated by DA signaling (Wise, 1982). Yet, this hypothesis does not agree with subsequent work that found that disruption of DA signaling with DA lesion and DA antagonists both attenuated reward-seeking but not reward-consumption behaviors (Blackburn et al., 1987; Ikemoto and Panksepp, 1996). These results suggest DA has a role specifically in appetitive behaviors rather than for its intrinsic hedonic value.

A popular hypothesis to account for the role of DA in reward-seeking behavior is the "incentive salience" model proposed by Berridge and Robinson (1998). Herein, the neural mechanisms of reward-seeking are broken up into two component parts, referred to as "liking" and "wanting." DA is thought to be involved in the "wanting" process and is responsible for the amount an animal is willing to work for reward. “Liking” on the other hand, is thought to be mediated by the hedonic response to a stimulus and is often determined by affective response upon

stimulus delivery. Indeed, subsequent work has shown that infusion of DA receptor antagonists to the NAcc increase the amount of time required for rats to approach and press a food-rewarded lever (Nicola, 2010). Furthermore, DA lesions to the NAcc will attenuate responding when a high number of lever presses are required to receive food reward (Aberman and Salamone, 1999). This effect of DA perturbations on motivation or “wanting” behavior further support the concept of incentive salience.

The use of FSCV has greatly enhanced understanding of DA signaling in the striatum and how it relates to "wanting.” For example, in a recent study, rats were selectively bred for interaction with a cue that was predictive of reward (sign-trackers) or for interaction with the receptacle where reward was delivered (goal-trackers) (Flagel et al., 2011). When DA was recorded from these animals with FSCV, DA increased with cue onset in sign-trackers only, thereby indicating DA was influencing motivation or "wanting" for the cue. Subsequent work has found that increased phasic DA release at the beginning of a sequential food-seeking task predicted more rapid completion of the task, thereby suggesting that DA is reflective of the incentive salience, or “wanting” of the rewarding stimulus (Wassum et al., 2012a).

Evidence from recordings of neuronal activity and DA release are only correlational. Direct causal evidence for a role of phasic DA in motivated behavior has been provided using stimulation of DA neurons and pharmacological manipulation of DA receptors. Infusions of DA receptor antagonists have been found to attenuate learning of a sequential food-seeking task shown to evoke

phasic DA release (Wassum et al., 2012a). Genetic knockout of the NMDA subtype of glutamate receptor has been found to ablate phasic DA release and to lead to deficits in some types of learning about rewarded behaviors (Zweifel et al., 2009). Phasic stimulation of DA neurons using optogenetics has been found to reactivate lever pressing for food reward (Adamantidis et al., 2011). Furthermore, this optogenetic activation of DA neurons has been found to induce learning about reward predictive cues and to increase responding (Steinberg et al., 2013). The evidence outlined above strongly implicates DA in mediating motivated behaviors. Therefore, it was of interest in this dissertation work to examine DA release using FSCV in rats during ethanol-seeking behavior.

Recordings of phasic DA with FSCV would add to the ethanol field

It is well established that ethanol increases the activity of DA neurons. For example, recordings of activity in dissociated DA neurons (Brodie et al., 1999b) and in behaving animals (Gessa et al., 1985) have observed an increase in DA neuron activity with administration of ethanol. Although the exact pharmacological mechanism for this excitation is still unclear, ethanol is thought to increase activity in DA neurons by enhancing activity of the hyperpolarization-activated cation (I_h) and blocking the small conductance calcium-activated potassium (SK) channels (Brodie et al., 1999a; Okamoto et al., 2006).

Given this electrophysiology work, a number of studies have focused on the effects of ethanol on DA release in the NAcc. Paradoxically, one of the first studies

to use FSCV to investigate the effect of ethanol on phasic DA release found that electrically stimulated DA was attenuated by doses of ethanol (Budygin et al., 2001). Another used FSCV to show that noncontingent (*i.e.,* investigator administered) doses of ethanol increased the frequency of phasic DA in the NAcc approximately 25 seconds after infusion of the drug (Cheer et al., 2007). A subsequent study found increases in tonic DA with microdialysis and increases in phasic DA with FSCV after a noncontingent infusion of ethanol (Robinson et al., 2009). While a robust increase in tonic DA was recorded, an increase in spontaneous phasic release events was only recorded in a subset of recording locations. Furthermore, a very recent study found phasic DA release in the dorsolateral striatum (DLS) and NAcc during operant ethanol self-administration (Shnitko and Robinson, 2014). Yet, in this report, recordings from the NAcc were collapsed between rats responding for a sucrose only solution and a sweetened ethanol solution, thereby limiting the conclusions that can be made from this study. Therefore, to date, there have been no investigations recording phasic DA in the NAcc during ethanol-seeking, despite the crucial role it may play in this behavior.

A number of studies using microdialysis in the NAcc have recorded increases in DA levels during anticipation for ethanol self-administration and during ethanol consumption (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003, 2005). Yet, due to the limited temporal resolution of dialysis, no investigations have been able to determine what specific components of ethanol self-administration are able to evoke DA release in the NAcc. Recording DA with FSCV in rats trained to self-administer ethanol would allow the

determination of the relationship between specific ethanol-seeking behaviors and phasic DA signaling, and was thus a major focus of this dissertation.

Recording with FSCV during ethanol self-administration is a technical challenge

In order to determine the role of DA in ethanol-seeking, rats must not be responding for any sucrose that may be added to the ethanol solution, nor responding to alleviate any caloric or liquid deprivation. To this end, the self-administration training required a 20% ethanol solution as a reward in rats that had *ad lib* access to food and water. It is critical that rats are not food or water restricted, because sucrose-seeking has been observed to result in phasic DA release (Roitman et al., 2004), and caloric restriction also has been shown to potentiate phasic DA signaling (Cone et al., 2014). Training rats to self-administer ethanol under these "replete" conditions while also performing the FSCV recording was a technical challenge. Recording DA in the NAcc with FSCV during ethanol self-administration has not yet been attempted and required the resolution of a number of technical challenges.

Dopaminergic contributions to ethanol-seeking behavior

Ventral striatum and dopamine in drug-seeking

The mechanisms underlying seeking of natural reinforcers are very similar to the mechanisms underlying drug-seeking. For example, one key finding suggesting that DA signaling during food-seeking is similar to DA signaling during

drug-seeking came with the observation that both delivery of a "Cheetos"-like snack to rats or drugs of abuse increase the release of DA in the NAcc (Di Chiara and Imperato, 1988), as measured by *in vivo* microdialysis. Food- and cocaine-seeking behaviors can be both be increased by electrical stimulation of DA neurons (Waldbillig, 1975; Phillips et al., 2003b). Furthermore, similar to findings in food-seeking, lesion of the DA inputs to the ventral striatum will attenuate cocaine self-administration (Roberts et al., 1980). Given these similarities, similar hypotheses for food-seeking have been applied to drug-seeking.

As DA has been implicated in incentive salience theory to be responsible for “wanting” (as reviewed above), researchers have linked DA to the incentive salience for drug (Robinson and Berridge, 2000). Indeed, nearly all abused drugs are thought to act directly or indirectly on DA signaling (Spanagel and Weiss, 1999). Some of the strongest evidence has come from imaging in human addicts during presentation of drug-associated cues. This work has led to the observation that DA increases in the striatum upon presentation of these cues, with the increase correlating with ratings of drug craving (Volkow et al., 2006). In animal studies, rats will work to self-administer DA receptor agonists directly into the NAcc (Carlezon et al., 1995). Furthermore, in a rodent model of compulsive drug-seeking wherein a rat must cross an electrified floor to receive cocaine (*i.e.,* drug-seeking persists despite negative outcomes), DA agonists infused into the NAcc increase cocaine seeking, whereas DA antagonists decrease the behavior (Saunders et al., 2013). In addition to mediating incentive salience for drug, DA has also been implicated in controlling the performance of the motor behavior during drug

seeking. For example, studies using FSCV have recorded phasic DA release events in the NAcc during cocaine self-administration (Phillips et al., 2003b). Together, these lines of evidence strongly suggest that DA is crucial for mediating both motivation and performance of drug-seeking behavior.

Ethanol's effect on the "wanting" or dopaminergic pathway

As is the case for natural and drug rewards, DA signaling in the NAcc has been strongly implicated in ethanol-seeking behavior, with persuasive evidence in the extant literature that ethanol results in potentiated DA signaling. Indeed, when rats are given low doses of ethanol, neural activity in the VTA increases (Gessa et al., 1985). These effects of ethanol on DA neuron activity may reflect a direct effect of ethanol on DA neurons in the VTA, because when VTA neurons are dissociated, put into culture and exposed to ethanol, an increase in firing also is observed when ethanol is applied to the culture (Brodie et al., 1999b). In microdialysis studies, systemic injection of ethanol results in dose-dependent increases in NAcc DA (Yim et al., 2000) and DA release evoked during self-administration is correlated to the amount of ethanol consumed (Gonzales and Weiss, 1998). FSCV studies have likewise revealed increases in DA in the NAcc upon noncontingent, systemic injection of ethanol (Cheer et al., 2007; Robinson et al., 2009). These findings have led to the theory that ethanol consumption leads to DA release due to ethanol's ability to increase DA neuron firing activity (as reviewed above). However, despite these findings showing an effect of ethanol on DA firing and release, studies using *in vivo* microdialysis have reported that extracellular concentrations of DA in the

NAcc increase independently of brain ethanol concentrations (Doyon et al., 2003, 2005), suggesting less of a pharmacological profile (*i.e.,* no dose-response curve) for the effects of ethanol on DA neuron function. Thus, in this dissertation work, I attempted to determine whether DA release evoked during ethanol-seeking is related to the amount of ethanol consumed.

Role of DA during ethanol self-administration

Many studies have clearly identified a role for DA during drug-seeking behavior, and evidence for a similar role for DA in ethanol-seeking has accumulated. Indeed, microdialysis studies during ethanol self-administration have reported increases in extracellular DA during the waiting period for ethanol and during ethanol consumption (Weiss et al., 1993; Melendez et al., 2002). Furthermore, administration of antagonists for DA receptors directly into the NAcc attenuates ethanol-seeking behavior (Czachowski et al., 2001). Similarly, increasing DA in the NAcc via local infusion of amphetamine potentiates ethanol-seeking behavior (Samson et al., 1999). Additionally, the VTA appears to be crucial in ethanol seeking, as studies have indicated that rats will self-administer microinjections of ethanol directly into the VTA (Gatto et al., 1994; Rodd-Henricks et al., 2000). These findings strongly suggest that increased DA signaling supports ethanol-seeking behavior.

Despite the findings cited above linking DA release to ethanol-seeking, not all of the literature agrees with this conclusion. Indeed, there are reports that administration of DA receptor antagonists is associated with increased (Levy et al.,

1991) or no effect on ethanol-seeking in rat models (Goodwin et al., 1996). In addition, inducing a hypodopaminergic state through ethanol withdrawal has been shown to be associated with increased ethanol-seeking behavior (Weiss et al., 1996). Thus, both hypo- and hyper-dopaminergia has been associated with increased ethanol-seeking. Given the disparity between these two states, the role of DA signaling during ethanol self-administration must be clearly elucidated.

Due to its role in motivating specific behavior during drug and reward seeking, many have hypothesized that DA is released in the NAcc during ethanol anticipation, operant behavior and consumption. In such studies, investigations that assessed extracellular DA levels via *in vivo* microdialysis during operant ethanol self-administration found that extracellular DA is increased while waiting for ethanol access and during ethanol self-administration (Weiss et al., 1993; Melendez et al., 2002). Yet, a subsequent study suggested that DA release evoked during the waiting period may be the result of handling and not specific to ethanol-seeking (Doyon et al., 2003). Also in these studies, self-administration included both bar pressing and ethanol consumption behaviors. A subsequent microdialysis study that was able to temporally separate these behaviors reported that DA release was evoked by ethanol consumption, but not lever pressing (Doyon et al., 2003). These findings conflict with several studies that show DA release during operant cocaine- and food-seeking behavior (Phillips et al., 2003b; Roitman et al., 2004), thereby suggesting that a similar signal would be present during ethanol-seeking. In this dissertation (Chapter 3), I investigate if DA signaling during ethanol-seeking occurs via similar mechanisms as other types of rewarding stimuli.

Due to the temporal limitations of *in vivo* microdialysis, these studies can show that extracellular DA levels increase during phases of ethanol seeking, but they do not allow for identification of which specific behaviors during ethanol seeking are associated with DA release. A major goal of this dissertation project, therefore, was to capitalize on the greater temporal resolution of the FSCV technique to determine whether DA release is evoked during specific ethanol-seeking behaviors. Specifically with this technique I attempt to address whether phasic DA release is evoked during anticipation of ethanol, lever pressing for ethanol reward and consumption of ethanol.

Habenular contributions to learning about the aversive outcomes of ethanol

Habenular neuroanatomy

The habenular complex is a component of a region of the brain called the epithalamus, which also includes the pineal body and the hypothalamus. The habenula is extremely conserved throughout evolution. Indeed, all vertebrates, including fish and reptiles, have a habenula (Concha and Wilson, 2001). While phylogenetic examination shows an enlargement of the habenula in more recently developed organisms, suggesting an increased importance of the region in behavior (Aizawa et al., 2011), the general conservation of this region across evolution is suggestive of a crucial role in survival and behavior.

The mammalian habenula is divided into two subregions: the medial (MHb) and the lateral habenula (LHb; Sutherland, 1982). The MHb receives input from

limbic regions, such as the septum. The LHb, on the other hand, receives input both from limbic regions, such as the lateral hypothalamus, and from basal ganglia nuclei, such as the internal segment of the globus pallidus (GPi; Herkenham and Nauta, 1977). The majority of afferents to the habenular complex passes through a tract called the stria medullaris. The output neurons of the habenula are exclusively glutamatergic in nature and mainly project through the fasiculus retroflexus to their targets (Nauta, 1958). Efferents of the MHb project mainly to the interpeduncular nuclei (Klemm, 2004). The efferents of the LHb, which are more relevant to reward-seeking behavior, project to the GABAergic rostromedial tegmental nucleus (RMTg), as well as to the serotonergic neurons in the dorsal and medial raphe, among other regions (Herkenham and Nauta, 1977; Quina et al., 2014). The RMTg has been implicated in robust control over DA neurons of the VTA (Jhou et al., 2009a). As DA release from the VTA has been strongly implicated in reward- and drug-seeking behaviors (see above), it then seems possible that the LHb has a role in these behaviors.

Functional role of the LHb in encoding value

Evidence for a role of the LHb in reward-seeking behavior has been recently found using electrophysiology studies in nonhuman primates. In an initial experiment, activity of the LHb was recorded in a task where a cue predicted either a small or large reward (Matsumoto and Hikosaka, 2007). Here, LHb activity was inhibited during the prediction or unexpected receipt of a large reward, but excited by the smaller reward. In a subsequent experiment, monkeys were trained on cues

that signaled a specific probability of delivery of either a rewarding juice delivery or an aversive air puff (Matsumoto and Hikosaka, 2009). Interestingly, LHb neurons were inhibited by cues that predicted a higher probability of reward and excited by cues that predicted the aversive stimulus. Furthermore, these investigators went on to identify projections to the LHb from the GPi as encoding reward-related stimuli (Hong and Hikosaka, 2008). Furthermore, in a human imaging study, activation of the habenula was observed if the person made an incorrect choice when predicting the motion of a moving object, suggesting a possible role in the encoding of prediction error seen in DA neurons (Ullsperger and von Cramon, 2003). These results suggest a role for the LHb in encoding value and therefore may instruct learning about these relationships.

The LHb is strongly implicated in learning about aversive stimuli

While the LHb has been implicated in both reward and aversion, evidence suggests that it may be more important in encoding aversion. Indeed, one of the first roles of the habenula to be identified was for mediating a behavioral response to aversive stimuli. In that seminal study, rats with a lesion of the habenula showed attenuated avoidance behavior for impending electric shock (Rausch and Long, 1974). More recent work has further solidified this role of the LHb by showing that a conditioned place aversion can be entrained by optogenetic stimulation of a subset of LHb neurons (Stamatakis and Stuber, 2012). Furthermore, electrical stimulation of the LHb leads to reduced sucrose-seeking, providing causal evidence that activity from this region can affect behavior by inducing aversion

(Friedman et al., 2011). As depression is characterized as a state of heightened aversion, it seems likely to be a link to the activity of the LHb. Indeed, in several models of depression, the amount of glucose metabolism as measured by 2-deoxyglucose (2-DG) was found to be elevated in the LHb (Caldecott-Hazard et al., 1988). Furthermore, LHb activity has been linked to depression in humans, with reports of deep brain stimulation (DBS) of an afferent to the LHb, the stria medullaris, treating a patient with intractable depression (Sartorius et al., 2010). Disruption of afferent input to the LHb with DBS is thought to attenuate its overall activity and lead to the reduced symptoms observed. Together, the above outlined evidence strongly suggests that the LHb is critical in mediating aversive learning.

The rostromedial tegmental nucleus strongly affects dopamine

The influence of the LHb on reward-seeking behavior may be secondary to its role in regulating DA neuron activity. Previous work has established that stimulation of the LHb can significantly attenuate activity of dopaminergic neurons of the VTA (Christoph et al., 1986) and that pharmacological inactivation of the LHb increases DA release in the NAcc as recorded by microdialysis (Lecourtier et al., 2008). A mechanism for this control of DA release by the LHb has come from discovery of the GABAergic RMTg. Indeed, recent evidence has shown that the majority of the influence of the LHb on VTA activity comes through the release of GABA from the RMTg (Jhou et al., 2009a). Literature from non-human primates (Hong et al., 2011) and rats (Lecca et al., 2011) provides evidence for a basal level of activity in the RMTg (10 Hz), suggesting that the RMTg provides tonic

suppression of VTA DA neurons. Similar to activity recorded from the LHb, the activity of the RMTg spikes when the rat is exposed to a highly aversive stimulus, such as the predator odor trimethylthiazoline (TMT) (Jhou et al., 2009a). Such activity would thus decrease the activity of VTA DA neurons. Conversely, when animals are given a rewarding natural stimulus, the activity of the RMTg drops (Hong et al., 2011), which would then be associated with increased activity of VTA DA neurons. Lastly, specific activation of neurons of the LHb that project to the RMTg have been found to entrain a conditioned place aversion (Stamatakis and Stuber, 2012). The above-outlined evidence provides a model (Figure 1.1) for how the LHb-RMTg-VTA pathway may mediate behavior towards both aversive and rewarding stimuli, similar to what is experienced during ethanol consumption.

The LHb is involved in drug aversion

Given the role of the LHb-RMTg-VTA pathway on aversion, it seems possible then that it may mediate learning about the aversive effects of abused drugs. Directly stimulating the LHb with DBS during cocaine self-administration has been shown to attenuate the behavior (Friedman et al., 2010). Another study has taken this finding further by identifying the LHb-RMTg pathway in the control of aversion to cocaine. In that case, i.v. infusion of cocaine initially causes a reduction in activity of the LHb, during which time cocaine appears to be rewarding. However, shortly thereafter, the administration of cocaine is associated with an increase during the time when animals appear to find the cocaine aversive (Jhou et al., 2013). This later increase in LHb activity is consistent with the activity seen

during the anticipation of aversive outcomes. In the same study, the experimenters found an increase in rats' speed to respond for an infusion of cocaine after an RMTg lesion, suggesting that the RMTg lesion lead to an attenuation of the rats' aversion to cocaine. A subsequent study further identified glutamatergic signaling in the LHb in mediating symptoms of cocaine withdrawal in mice, hereby further reinforcing the idea that increased LHb activity is associated with aversion (Meye et al., 2015). Finally, other regions of the habenula have been implicated in control of aversion to drugs of abuse. For example, in the case of nicotine, pharmacological inactivation of the MHb was found to increase nicotine self-administration, indicating a role for the MHb in aversion (Fowler et al., 2011). Taken together, the above evidence strongly suggests that the habenular complex, and the LHb in particular, may be involved in aversion to drugs of abuse.

Possible role of the LHb in ethanol-seeking

Given the role of the LHb in aversion to drugs of abuse, it is plausible that it mediates aversion to ethanol. The only evidence to date in support of this hypothesis, however, is from studies using 2-DG to determine changes in cellular metabolism after ethanol administration. Consistent with the hypothesis above, these studies have found a marked increase in the metabolism of cells in LHb in association with noncontingent injections of ethanol injections (i.p.; Williams-Hemby and Porrino, 1994), or in a self-administered, ethanol-drinking paradigm (Williams-Hemby et al., 1996). A major goal of this dissertation, therefore, was to determine what role, if any, the LHb has in ethanol-seeking behavior in rats.

Identifying anatomical regions and their role in ethanol-seeking will enhance our understanding of ethanol-seeking and assist in the development of possible therapeutics.

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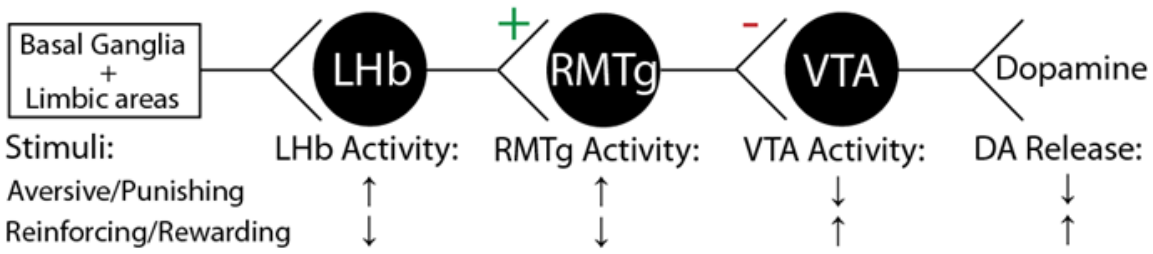

Figure 1.1 Circuitry relationships

A representation of the circuitry relationships in the proposed pathway and the activity patterns associated with contextual stimuli.

CHAPTER 2

DEVELOPMENT OF FAST SCAN CYCLIC VOLTAMMETRY AS A TECHNIQUE TO RECORD SUBSECOND DOPAMINE IN THE NUCLEUS ACCUMBENS DURING OPERANT ETHANOL SELF-ADMINISTRATION

Abstract

Subsecond, phasic dopamine release in the nucleus accumbens is critical to motivating performance of reward and drug-seeking behavior. An essential technique used to record phasic dopamine is fast scan cyclic voltammetry. Despite the crucial role of phasic dopamine signaling in reward and drug-seeking, the role of phasic dopamine during ethanol-seeking remains poorly understood. The difficulty of inducing operant ethanol-seeking in rat models, and performing behavioral recording from a tethered animals make the technique difficult to master. To address this issue, experimental methods to train rats to self-administer ethanol at robust levels without caloric restriction and to record phasic dopamine during this behavior were necessary.

In order to develop the necessary techniques for recording phasic dopamine in rats with *ad lib* access to food and water, I first conducted a series of pilot

studies. Three distinct studies were performed in ascending order of anticipated difficulty and consisted of recording phasic DA release evoked by: electrical stimulation, classical conditioning, and during operant ethanol self-administration with caloric restriction. Quantification of dopamine during these conditions suggest that the methods used were sufficient record phasic dopamine during ethanol self-administration in rats with *ad lib* access to food and water.

Introduction

Dopaminergic signaling from in the nucleus accumbens (NAcc) has long been implicated in reward and drug-seeking behavior (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Wise, 2004; Redgrave et al., 2010). Dopaminergic neurons in the ventral tegmental area (VTA) have two distinct firing modes, leading to tonic and phasic dopamine (DA) release (Grace, 1991). Tonic DA release is driven by intrinsically generated, low frequency firing (1-5 Hz) (Grace and Bunney, 1983; Johnson and North, 1992; Nedergaard and Greenfield, 1992). This is thought to result in DA concentrations (5-20 nM) in the NAcc that change with a time scale on the order of minutes (Parsons and Justice, 1992; Hogan et al., 1994). Phasic activity results from high frequency firing that occurs in a high frequency burst (14-30 Hz) (Grace and Bunney, 1984; Hyland et al., 2002). Phasic release causes a robust increase in DA concentration (as high as 1 μM) on a subsecond timescale (Garris et al., 1997). This increase is thought to activate a subset of lower affinity DA receptors (Gonon, 1988; Goto and Grace, 2005).

Recordings of phasic DA release during cocaine- and food-seeking

behavior are consistent with theories suggesting phasic DA mediates this behavior (Phillips et al., 2003b; Roitman et al., 2004). These findings are supported by findings where exogenous stimulation of phasic DA release increases cocaine- and food-seeking (Phillips et al., 2003b; Adamantidis et al., 2011; Steinberg et al., 2013). Lastly, a selective disruption of phasic DA attenuates learning about both rewarding and aversive outcomes (Zweifel et al., 2009). These findings suggest that phasic DA is essential to understanding reward and drug-seeking, thereby suggesting a possible role in ethanol-seeking.

Traditional techniques to record dopaminergic signaling *in vivo* include microdialysis to detect neurotransmitter release in the region of interest or electrophysiology to extracellularly record action potentials. Microdialysis requires implantation of probes made out of semipermeable membrane to an anatomical region of interest (Westerink, 1995; Plock and Kloft, 2005). Analyates, such as extracellular DA that are present in the extracellular space can diffuse through the membrane and thereby enter the microdialysis probe (Watson et al., 2006). The analytes within the probe are then carried by a flow of artificial cerebrospinal fluid to a collection vial for chromatographic analysis performed off-line. The strength of this technique is that is possible to record the concentration of a wide variety of neurotransmitters with high sensitivity (Perry et al., 2009). However, the temporal resolution of microdialysis is limited, as measurements typically occur on a timescale of minutes therefore making the technique unable to resolve DA release evoked by a specific behavior (Watson et al., 2006; Schultz, 2007). Despite recent improvements in temporal resolution, the temporal resolution afforded by

microdialysis is not sufficient to identify individual phasic DA transients (Floresco et al., 2003).

Electrophysiological techniques, on the other hand, have a very high temporal resolution and therefore can record high frequency bursts of action potentials from individual dopaminergic neurons (Lewicki, 1998). However, VTA DA neurons project to a variety of target regions, which have idiosyncratic DA release properties (Swanson, 1982; Margolis et al., 2008). Furthermore, recent evidence suggests that electrophysiological features of dopaminergic and GABAergic neurons in the VTA are not readily distinguishable (Margolis et al., 2006, 2008). These technological limitations limit the utility of extracellular recording techniques for measuring DA signaling contributing to motivated behaviors.

Fast scan cyclic voltammetry (FSCV) has temporal resolution sufficient to record DA transients (Robinson et al., 2003). This technique takes advantage of the electroactive properties of catecholamines to take measurements of DA, norepinephrine (NE) and serotonin (5-HT) concentrations in an anatomical region of interest with a time resolution of 100 ms (Phillips et al., 2003a). In addition, the electrode used in FSCV is compact (150 μm in length), allowing recording from very specific anatomical brain regions and can be chronically implanted (Clark et al., 2010). While powerful, the technique has limitations that must be noted. First, while 5-HT can be uniquely identified, FSCV cannot distinguish between DA and NE (Palij and Stamford, 1993). This necessitates electrode implantation in regions that are either predominantly dopaminergic or noradrenergic (Park et al., 2009).

Also, FSCV cannot record 5-HT simultaneously during recordings of either DA or NE (Jackson et al., 1995). Although recent advances electrode technologies may resolve this limitation, these have not seen wide adoption (Swamy and Venton, 2007). Furthermore, successful recordings of other neurotransmitters have been reported, these techniques are not yet widely available (Wassum et al., 2012b). Despite these limitations, FSCV technology is a critical tool as it is currently the only method available to resolve DA transients in a specific brain region during behavior.

To date, FSCV has not be used to characterize DA signaling during ethanol-seeking. Likely contributing to this paucity of research is that ethanol-seeking in the rat is difficult to acquire and maintain without the use of sweeteners (Samson, 1986) or caloric restriction (Meisch and Thompson, 1974). Several existing studies have recorded phasic DA after noncontingent injection of ethanol. These studies found a robust increase in phasic DA release evoked by ethanol infusion (Cheer et al., 2007) and an increase in spontaneous DA transients (Robinson et al., 2009). Another study recorded phasic DA during self-administration in the dorsolateral striatum but used sucrose to enhance responding (Shnitko and Robinson, 2014). Responding for sucrose alone is well known to lead to phasic DA release (Roitman et al., 2004). Therefore, the presence of sucrose in ethanol is likely to obscure ethanol-evoked release.

Our lab has successfully induced robust operant responding for 20% ethanol solution (Haack et al., 2014). This report used a paradigm of intermittent ethanol access (IEA), where ethanol is given for three, 24 hour periods during a

given week. IEA has been show to induce robust ethanol consumption in home cage and induce operant self-administration without the addition of sweeteners (Simms et al., 2008, 2010). While effective in voluntary operant responding, the utility of this approach for recording *in vivo* was unproven. In particular, tethering required for *in vivo* recording could diminish operant responding. To mitigate this concern, I used a recently developed version of the FSCV technique that allows the chronic implantation of electrodes (Clark et al., 2010). Use of acute electrodes requires fresh electrodes to be lowered into position on each day of recording with a micromanipulator (Phillips et al., 2003a). This approach requires larger headcaps to accommodate the micromanipulator and considerable handling to position the electrode before the experimental session. Both large headcaps and the process of electrode insertion with a micromanipulator are likely to induce a stress response in an awake rat. Given that stressful stimuli has long been known to result in attenuation of ethanol self-administration in rats (Ng Cheong Ton et al., 1983), this makes acute electrodes less than ideal for these studies. The combination of the recent development of ethanol self-administration training techniques and chronic FSCV electrodes thus offered a promising route for recording phasic DA during this behavior.

To establish FSCV recording, I conducted pilot FSCV experiments incorporating increasingly challenging technical steps. The first of these attempted to evoke DA release via electrical stimulation of the VTA. Electrical stimulation of this region is known to evoke robust DA release in both behaving (Phillips et al., 2003a) and anaesthetized rats (Millar et al., 1985). Given the low sensitivity and

lack behavioral performance needed to perform stimulation experiments, I identified VTA stimulation in behaving rats as the most achievable initial experimental milestone. Second, I aimed to record DA signal in a classical conditioning paradigm. The presentation of a reward predictive cue in a classical conditioning paradigm has been shown to result in a large amount of cue-evoked DA release (Day et al., 2007). This large DA signal, while considerably lower than stimulation-evoked DA release, gives the greatest probability of recording cue evoked DA release. Third, I attempted to record DA during operant self-administration while motivation for ethanol was enhanced with food deprivation. Caloric restriction has been shown to potentiate phasic DA for food reward (Cone et al., 2014). Furthermore, caloric restriction has also been shown to boost ethanol consumption (Meisch and Thompson, 1974). This combination gives the greatest probability of recording DA during ethanol-seeking while also yielding enough operant responses to be behaviorally relevant. Successful completion of these milestones increased the chance of success during subsequent experiments that will record phasic DA during operant ethanol self-administration without caloric restriction.

Methods

Animals

Male Sprague-Dawley rats (250 g, Charles River) were used in all experiments. Rats undergoing VTA stimulation (n = 31 rats) had *ad lib* access to food and water throughout the experiment. Rats in the classical conditioning (n =

36) and ethanol-seeking (n = 5) experiments were calorie restricted to 90% of initial body weight. In the ethanol-seeking experiment, rats were trained to consume ethanol using an IEA paradigm in the home cage until they reached criterion levels of voluntary ethanol intake (see details below). After training these rats were implanted with FSCV electrodes for DA detection.

Stereotaxic FSCV electrode implantation

Implantation of FSCV electrodes were performed while the rat was under isoflurane anesthesia (5% for induction and 2% for maintenance in O_2). The scalp of the rat was first shaved and placed in the stereotax. The scalp was prepared for surgery by swabbing with ethanol, followed by betadine (10%, Purdue Frederick, Stamford, CT). An incision exposed the cranium and the surface was cleaned with hydrogen peroxide. Burr holes were made with a dental drill to be positioned above the NAcc core (AP: 1.3, ML: 1.3, DV: 7.2) and a region of contralateral forebrain. In-house fabricated carbon fiber and Ag/AgCl electrodes were lowered into the NAcc and contralateral forebrain, respectively (Clark et al., 2010). For experiments involving VTA stimulation, burr holes where made above the VTA (AP: -4.9, ML: 0.8, DV: 8.4) and a bipolar stimulating electrode (PlasticsOne, Roanoke, VA) was lowered. Electrodes, wiring and a datamate connector were encased in dental cement and anchored to the skull with 4 skull screws. A dose of penicillin (6000 U/kg IM, VetOne, Boise, ID) and buprenorphine (0.1 mg/kg IP, Reckitt Benckiser, Richmond, VA) were given postsurgery for suppression of infection and analgesia, respectively.

Fast scan cyclic voltammetry

Each voltammetric scan consists of a linear ramping of the carbon fiber electrode's potential relative to the Ag/AgCl electrode from -0.4 V to 1.3 V. The period of increasing potential is called the anodic sweep. After the voltage of the carbon fiber electrode reaches 1.3 V, the voltage is ramped from 1.3 V to -0.4 V, the period of decreasing potential is called the cathodic sweep. Once -0.4 V is reached, the potential of the electrode is held at -0.4 V until the next voltammetric scan that occurs in 91.5 ms. Each voltammetric scan takes 8.5 ms and rate of voltage ramping runs at slew rate of 400 V/sec (Figure 2.1A). DA on the surface of the electrode is oxidized into dopamine-o-quinone during the anodic sweep. This oxidation reaction causes a donation of electrons to the carbon fiber electrode. During the cathodic sweep the dopamine-o-quinone on the surface of the electrode is reduced back into dopamine by a donation of electrons from the electrode to the reaction (Figure 2.1B). The quantities of electrons gained and lost to the electrode are proportional to the quantity of DA molecules oxidized and reduced from the voltammetric scan. Gain and loss of electrons from this reaction to the electrode are recorded as negative and positive current, respectively, in the data acquisition software. The oxidation of DA during the anodic sweep occurs when the carbon fiber electrode is between 0.6 and 0.7 V and the reduction of the dopamine-o-quinone occurs when the electrode is between -0.2 and -0.3 V during the cathodic sweep (Figure 2.2). A peak of positive current at the oxidation voltage and a peak of current at the reduction voltage identifies DA. The recorded current is amplified by a custom-built headstage and routed through an electrical commutator (Crist

Instruments, Hagerstown, MD) to allow free movement of the rat. Generation of the voltammetric waveform and data collection was performed with custom software coded in LabVIEW for PC-based data acquisition hardware (National Instruments, Austin, TX).

During all experiments, rats were placed in a testing apparatus that was enclosed by a faraday cage to minimize electrical noise. Before beginning data acquisition, the electrode was cycled at a frequency of 60 Hz to equilibrate the electrode prior to DA detection. During recordings, the recorded current was background subtracted from a 10 scan average at the beginning of the trial. A band-pass filter was applied (0.025-2000 Hz) and smoothed with a 5 scan running average. Chemometric analysis was used to isolate DA from other recorded analytes (Heien et al., 2004). Cyclic voltammograms of pure DA, pH shift and electrode drift were included in the training set that was used to entrain the chemometric analysis (Clark et al., 2010). To identify DA, the recorded signal must exceed 2.5 times the root mean square of noise and must occur in a window of 200 ms centered to the onset of the stimuli expected to evoke DA release.

Electrically evoked DA release

Rats that received stimulation electrodes to the VTA were given a 4 week period between electrode implantation and testing. Stimulation parameters were chosen based on previous studies establishing current parameters sufficient to evoke robust DA release (120 μA, 60 Hz, 24 pulses; Heien et al., 2005). Delivery of electrical stimulation was performed with a stimulator in constant current mode

(AM Systems, model 2200, Sequim WA). Custom coded software timed stimulation pulses so that FSCV scans and the pulses were interleaved. The avoidance of simultaneous stimulation pulse and FSCV scan prevented stimulation artifact in the FSCV recordings.

Classical conditioning of sucrose pellet delivery

After FSCV electrode implantation, rats were given a 4 week period before behavioral training. Prior to training rats were food deprived until body weights were below 90% of baseline. Upon reaching the requisite body weight, rats were trained in the classical conditioning paradigm. Here, a 1 kHz tone sounded and a cue light was illuminated 5 seconds before the delivery of a sucrose pellet. Presentations of the conditioned stimulus (CS) were separated by an intertrial interval of 2 minutes. Rats were trained with a 1 hour training session prior to recording sessions. At least 1 day after initial training, rats were tethered to the FSCV recording apparatus. If rats did not reach the food magazine 1 second after pellet delivery, FSCV recordings were performed for an additional recording day. Prior to beginning recording on the classical paradigm, rats were given free sucrose pellet delivery at a randomized interval (mean 2 minutes) with no predictable cue. Data from the classical session was only analyzed if DA release was evoked upon receipt of sucrose pellet. For analysis of the CS stimulus trials, data analysis was centered to the onset of the CS.

Operant ethanol-seeking after caloric restriction

The IEA paradigm has been shown to lead to robust ethanol consumption in the home cage and lead to robust self-administration (Simms et al., 2008, 2010). Rats were given a minimum of 5 weeks access to IEA to reach a criterion levels of ethanol intake: greater than or equal to 3 g/kg/24 hrs. After reaching this criterion they were advanced to the next phase of the study. Once minimum intake levels were reached, rats were food deprived to 90% of original body weight and trained on an operant ethanol self-administration paradigm in standard operant training chambers. During this training, a cue light came on above the active lever, 1 second later the active lever extended. One second after lever press, 20% ethanol was delivered into a cup. Lever presses were rewarded with ethanol on a FR1 schedule of reinforcement. Rats were trained during overnight sessions and needed to meet a criterion of 40 lever presses in a 16 hour session. All training occurred with a 24 hour break between sessions. After meeting criterion, rats were transitioned using the same operant paradigm while tethered to and recorded on the FSCV testing apparatus.

Statistics

Changes in DA concentrations in ethanol-seeking experiments were analyzed during cue presentation (extension of the operant response lever), during operant responding (lever press) and consumption of ethanol (head entry into reward receptacle). In each case, DA concentrations were analyzed in 500 ms bins. Changes in DA concentration were quantified by comparing DA

concentrations in the 500 ms bin after the behavior to baseline levels. Comparisons of ethanol predictive cues were analyzed using a one-way repeated measures ANOVA with Bonferroni correction. Comparisons of lever press and consumption DA used a paired t-test. All statistical analysis was performed in SPSS software (IBM, Armonk, NY). As stimulation and classical conditioning experiments were proof-of-concept replications of already published work, statistical analysis was not performed for these studies.

Results

VTA stimulation results in robust DA release

In a freely-moving rat, phasic DA was recorded upon onset of electrical stimulation of the VTA (t = 0; Figure 2.3). In the presented trial, DA signal increased just after the onset of VTA stimulation (purple/green signal in psuedocolor heat plot, Figure 2.3, top), and peaked approximately 1.7 seconds after stimulation onset with a peak change in current recorded as 2.2 nA and -0.5 nA for the oxidative and reductive potentials, respectively. Consistent with the electrochemical signature of DA, oxidization of the analyte occurred near 0.67 V, with reduction occurring at -0.25 V, as highlighted in the cyclic voltammogram (Figure 2.3, inset). Calculated DA concentration at t = 1.7 seconds increased 142.8 nM from baseline recorded at t = -5 seconds (Figure 2.3, bottom, red trace). DA concentrations remained elevated for over 10 seconds beyond the onset of electrical stimulation. Levels of pH gradually became more basic at the recording site once electrical stimulation was delivered. Due to the constant increase in pH,

the peak change in pH occurred at the end of the recording session, 10 seconds after the onset of electrical stimulation (pH = 0.8; Figure 2.3, bottom, green trace) but likely persisted beyond the length of the recording period. Residual values remained low (peak Q = 11.6) throughout the recording period and therefore well below the 95% confidence interval (Q_α = 196).

Presentation of a reward-paired CS results in robust DA release

Upon training with a classical conditioning paradigm, the presentation of a CS paired to reward resulted in phasic DA for the onset of the CS rather than reward receipt (Figure 2.4). In the presented trail, DA signal increased just after the onset of the CS (t = 0, purple/green signal in psuedocolor heat plot, Figure 2.4, top), and peaked approximately 0.5 seconds after stimulus onset. Consistent with the electrochemical signature of DA, oxidation of the analyate occurred near 0.65 V as shown in the cyclic voltammogram (Figure 2.4, inset). Calculated DA concentration at t = 0.5 seconds increased 41.2 nM from the baseline recorded at t = -2 seconds (Figure 2.4, bottom, red trace). Levels of basic pH shift occurred approximately 1.8 seconds after the onset of the CS and peaked at t = 4.5 seconds (Figure 2.4, bottom, green trace).

DA release is evoked for ethanol seeking and consumption
with food-deprivation

In a food-deprived rat trained to lever press for ethanol reward, the occurrence of the lever press evokes phasic DA release in the NAcc (Figure 2.5).

In the presented trial, DA signal increases and peaks just prior to when lever press occurs (t = 0, purple/green signal in psuedocolor heat plot, Figure 2.5, top). Consistent with the electrochemical signature of DA, oxidation of the analyate occurred near 0.7 V, as shown in the cyclic voltammogram (Figure 2.5, inset). Calculated DA concentration at t = -0.6 seconds increased 27 nM from the baseline recorded at t = -3 seconds (Figure 2.5, bottom, red trace). Basic pH shift occurred approximately at t = -0.3 seconds and peaked 1 second after lever press (Figure 2.5, bottom, green trace).

When the mean DA concentration was calculated from all analyzed trials in all experimental animals (n = 4 rats) no significant increase in DA was observed upon the presentation of cues that are predictive of ethanol (Figure 2.6A). While there was a ramping increase after light presentation and a rapid peak after lever presentation, neither of these increases are significant compared to baseline DA (Figure 2.6B). While predictive cues did not evoke DA release, an increase in phasic DA was associated with instrumental responding for ethanol (Figure 2.7A). Mean DA concentrations increased 1 second prior to lever press and peaked 0.1 seconds after the occurrence of the lever press. This increase was found to be statistically significant compared to a period of baseline (Figure 2.7B, $p < .05$) In addition, during the same experimental trials consumption of ethanol was found to be associated with phasic DA release (Figure 2.8A). Mean DA levels increased when the rat entered the reward receptacle (t = 0) and peaked 0.8 seconds after the occurrence of reward receptacle entry. This increase was found to be statistically significant compared to a period of baseline (Figure 2.8B, $p < .05$).

Discussion

While DA signaling has been an area of intense study, our understanding of the dynamics of DA signaling during ethanol-seeking remains incompletely understood. This lack of data necessitates the development of techniques to record rapid changes in DA concentration during ethanol-seeking. Here, I report that phasic DA release was successfully recorded upon electrical stimulation, presentation of a CS after classical conditioning and calorically-motivated ethanol-seeking. These results demonstrate that FSCV can reliably record physiologically evoked phasic DA release during behavior. Demonstration of recording with this methodology enables the use of the same techniques in recording during ethanol-seeking without the use of caloric restriction.

Stimulation of the VTA has long been known to result in robust and rapid increases in DA concentrations (Millar et al., 1985). While recordings of DA release evoked by electrical stimulation can range into μM concentrations, many of these recordings use acute, pulled glass electrodes rather than the chronic electrodes described here (Phillips et al., 2003a). My recordings of DA after stimulation were within the normal range for electrical stimulation in awake and behaving animals using electrodes of this type (Clark et al., 2010). The chronic implantation of the stimulating and recording electrodes likely results in a degree of glial encapsulation at the electrode and thereby may attenuate the recorded signal (Clark et al., 2010). Another contributing factor to the relatively small magnitude of the electrically stimulated DA signal is that my approach did not allow the optimization of the recording location (Wightman et al., 2007). While recording electrically stimulated

DA has led to a greater understanding of dopaminergic signaling, understanding of physiological DA requires recording DA transients evoked by environmental stimuli.

Physiological DA release results in much smaller increases in DA concentrations than those resulting from electrical or optogenetic stimulation (Millar et al., 1985; Phillips et al., 2003a; Tsai et al., 2009). Here, I completed FSCV recording using a behavioral paradigm that has been shown to evoke robust concentrations of physiological DA (Day et al., 2007). The choice of paradigm was made to maximize my chances of detecting phasic DA release, given the small magnitude of physiological DA release. In my data, DA release was evoked for cues that were predictive of the rewarding stimuli, rather than the receipt of the reward itself. This pattern of DA release matched closely with theories that suggest DA is acting as a signal of reward prediction error. In these theories, DA signaling that initially occurs for reward receipt will become transferred to cues that are predictive of reward receipt (Ljungberg et al., 1992; Schultz et al., 1997). The demonstration of my ability to record physiological DA signals, indicates that my methodology is refined enough to sufficiently record DA release during operant responding for reward.

Cues that were predictive of ethanol availability were not sufficient to evoke phasic DA release in my specific behavioral paradigm. This result was surprising as work from our lab and others have recorded DA transferring to more temporally distal cues with training in paradigms of food-seeking (Ljungberg et al., 1992; Schultz et al., 1997; Day et al., 2007; Wassum et al., 2012a). Furthermore,

microdialysis studies have observed a marked increase in DA during a waiting period prior to ethanol self-administration (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002). However, a subsequent study suggested this may be the result of handling and is not specific to ethanol-seeking (Doyon et al., 2003). One possibility for the lack of signal is that there are two, temporally separated predictive cues in the behavioral paradigm. Separate cues may lead rats to associate different cues with ethanol, thereby splitting the recorded signal. Future studies combining the predictive cues to ensure the presence of a single, powerful predictive stimulus might result in more robust DA signal.

I found that operant ethanol-seeking behavior evoked phasic DA release in food-deprived rats. During this behavior, DA increased prior to and peaked just after the occurrence of lever press. Prior microdialysis studies have not recorded increases in DA during periods where rats must lever press many times for ethanol reward (Doyon et al., 2003). While my findings do not agree with the microdialysis literature, the increase in DA is similar to results from FSCV recordings of rats engaged in operant lever pressing for food or cocaine reward (Phillips et al., 2003b; Roitman et al., 2004). The demonstration of DA during operant responding for ethanol suggests a role for DA signaling during operant responding for a variety of reinforcing stimuli.

In addition, I found in these experiments that consumption of ethanol was also sufficient to evoke DA release. Ethanol consumption evoked a DA signal similar to that reported after receipt of a food reward (Day et al., 2007; Brown et al., 2011; Cone et al., 2014). The presence of this signal suggests that ethanol can

act as a reinforcing stimulus in rats that have extensive experience with ethanol consumption. Furthermore, my results agree with prior microdialysis studies that show a substantial increase in DA levels during periods of ethanol consumption (Doyon et al., 2003, 2005). The release of DA has been hypothesized to occur for cues that predict reward (Ljungberg et al., 1992; Schultz et al., 1997). It seems possible then, that the taste of ethanol acts as a cue to predict the increased activity of dopaminergic neurons that occur through ethanol's pharmacological profile after intake.

A caveat to my data showing DA release during operant responding and during ethanol consumption was the induction of food-deprivation prior to recording. Recordings during ethanol-seeking are very similar to signal recorded during food-seeking (Roitman et al., 2004; Brown et al., 2011; Cone et al., 2014). This suggests that the recorded DA signals may be related to the caloric rather than the pharmacological effects of ethanol on dopaminergic neurons. Indeed, food deprivation has been shown to potentiate phasic DA signals for food receipt through ghrelin signaling (Cone et al., 2014). As drinking in humans is likely motivated by ethanol's rewarding pharmacological effects rather than its caloric value (Dole et al., 1985; Kraus et al., 2005), performing this study in rats with *ad lib* access to ethanol would be more relevant to human behavior. In Chapter 3, I describe a study where rats with *ad lib* access to food and water self-administer ethanol using a similar paradigm as described here.

In all of the recordings presented, DA release is followed by a basic pH shift of varying magnitudes. This finding is consistent with findings from FSCV studies

that observed comparable pH shift from electrical stimulation and physiological DA release (Cheer et al., 2006; Ariansen et al., 2012). The pH shift after DA likely arises from neural activity in that brain region (Magnotta et al., 2012). Neural activity increases rates of glucose metabolism, leading to increased CO_2. The immediate result of neural activity is increased blood flow, leading to increased CO_2 clearance and levels of O_2 (Venton et al., 2003). This reduction in CO_2 concentrations leads to a basic pH shift. Blood oxygen level dependent (BOLD) imaging also records blood oxygenation levels (Ogawa et al., 1990). Studies using BOLD in humans and primates have found similar changes in blood oxygenation in the NAcc after rewarding stimuli (Haber and Knutson, 2010).

Changes in pH can have a drastic effect on the cyclic voltammograms recorded during FSCV and can interfere with measurements of DA. The magnitude of the pH shift is very unpredictable, and is likely due to the proximity of the carbon fiber electrode to blood vessels, with closer electrodes more affected by pH shifts (Venton et al., 2003). To prevent pH from affecting my recordings, pure cyclic voltammograms of DA, pH and electrode drift of increasing magnitude were used to entrain a chemometric analysis (Clark et al., 2010). This analysis was used to isolate signal that resulted from changes from DA concentration rather from pH shift or electrode drift. In chemometric analysis, residual values (Q) are the elements of signal that cannot be accounted for by the model. In my data, the model would be the pure voltammograms of DA, pH and electrode drift included in a training set for the chemometric analysis. Another residual value (Q_α) is calculated to determine the 95% confidence interval that the provided model can

explain experimental data (Keithley et al., 2009). Values of Q that exceed Q_α are deemed to contain signal that cannot be accounted for by the training set with 95% confidence and are removed during analysis (Heien et al., 2005). In the data in this report and data in Chapter 3, values of Q for all the cyclic voltammograms used never exceeded the calculated Q_α value.

My data demonstrate robust recordings of both electrically stimulated and physiological DA release with FSCV. In addition, I developed a training program that successfully led to operant ethanol self-administration during FSCV recordings. In these experiments, I collected preliminary data suggesting that operant ethanol-self administration and ethanol consumption can evoke phasic DA release. Such findings are significant because it is one of the first reports showing the temporal dynamics of DA release in the NAcc during ethanol-seeking. To address this question, recordings must be made from rats with *ad lib* access to food and water, along with using a compound cue to signal the availability of ethanol. An investigation making these changes to the experiment will be described in Chapter 3.

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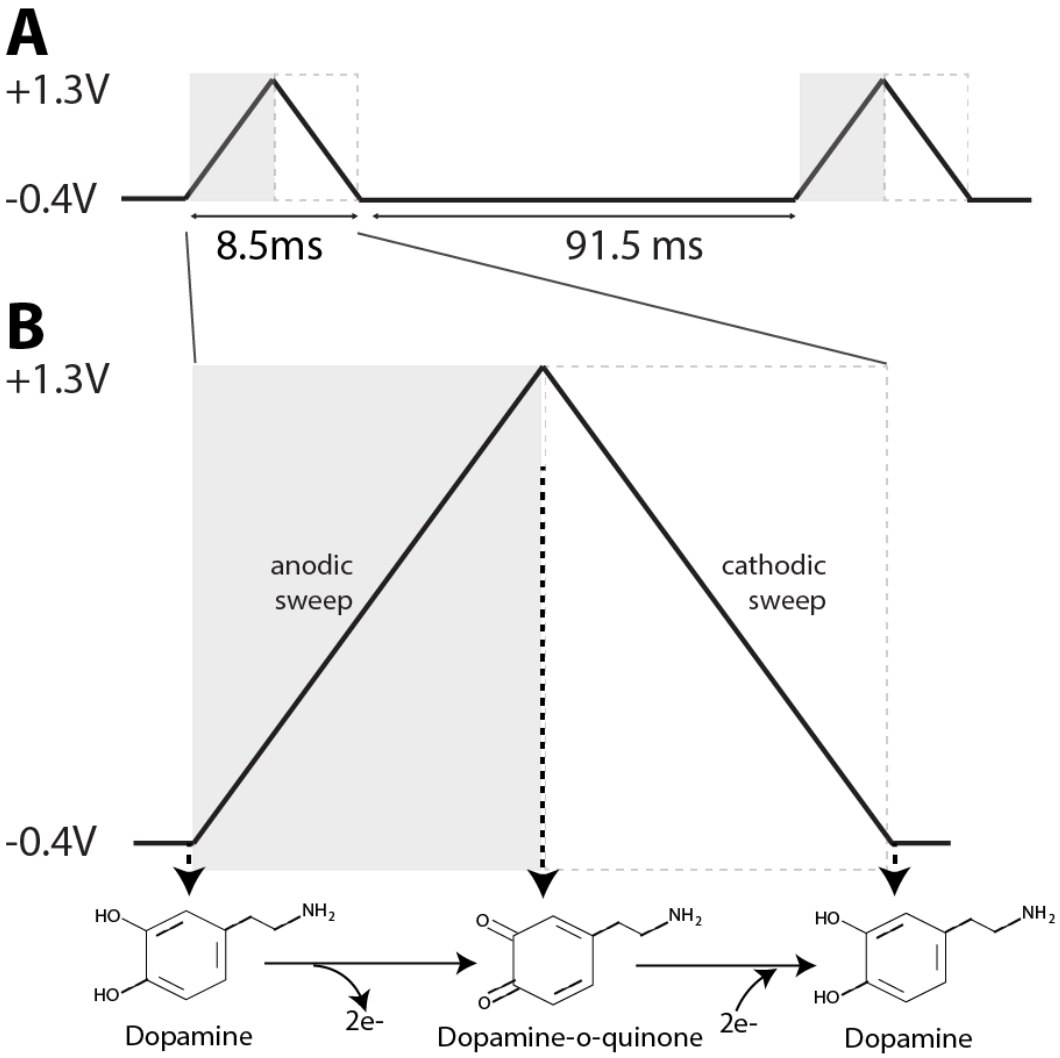

Figure 2.1. Oxidization of DA on the surface of the carbon fiber microelectrode

A, A voltammetric scan is run every 100 ms. Each entire scan takes 8.5 ms and there is a delay of 91.5 ms before the next scan begins. ***B***, DA binds to the carbon fiber electrode at -0.4 V. Increasing the voltage in the anodic sweep causes DA to be oxidized into dopamine-o-quinone and the reaction donates 2 electrons to the carbon fiber electrode. Decreasing the voltage in the cathodic sweep causes dopamine-o-quinone to be reduced into DA and 2 electrons are gained from the carbon fiber electrode. (Adapted from: Arnold et al., 2015)

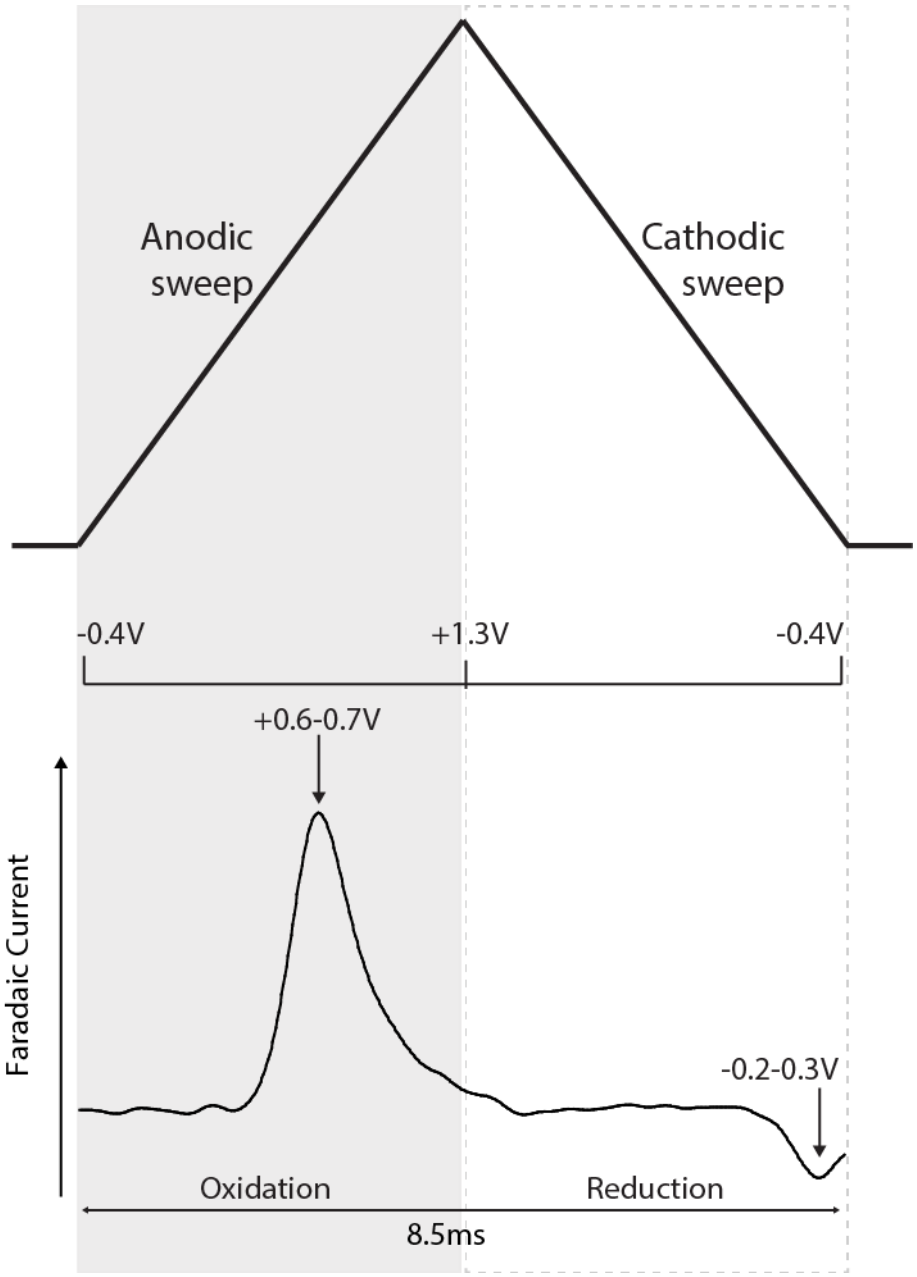

Figure 2.2. DA signature

Signature identifying the presence of DA by oxidization (positive current at 0.6-0.7 V) and reduction (negative current at -0.2 - -0.3 V) (Adapted from: Arnold et al., 2015)

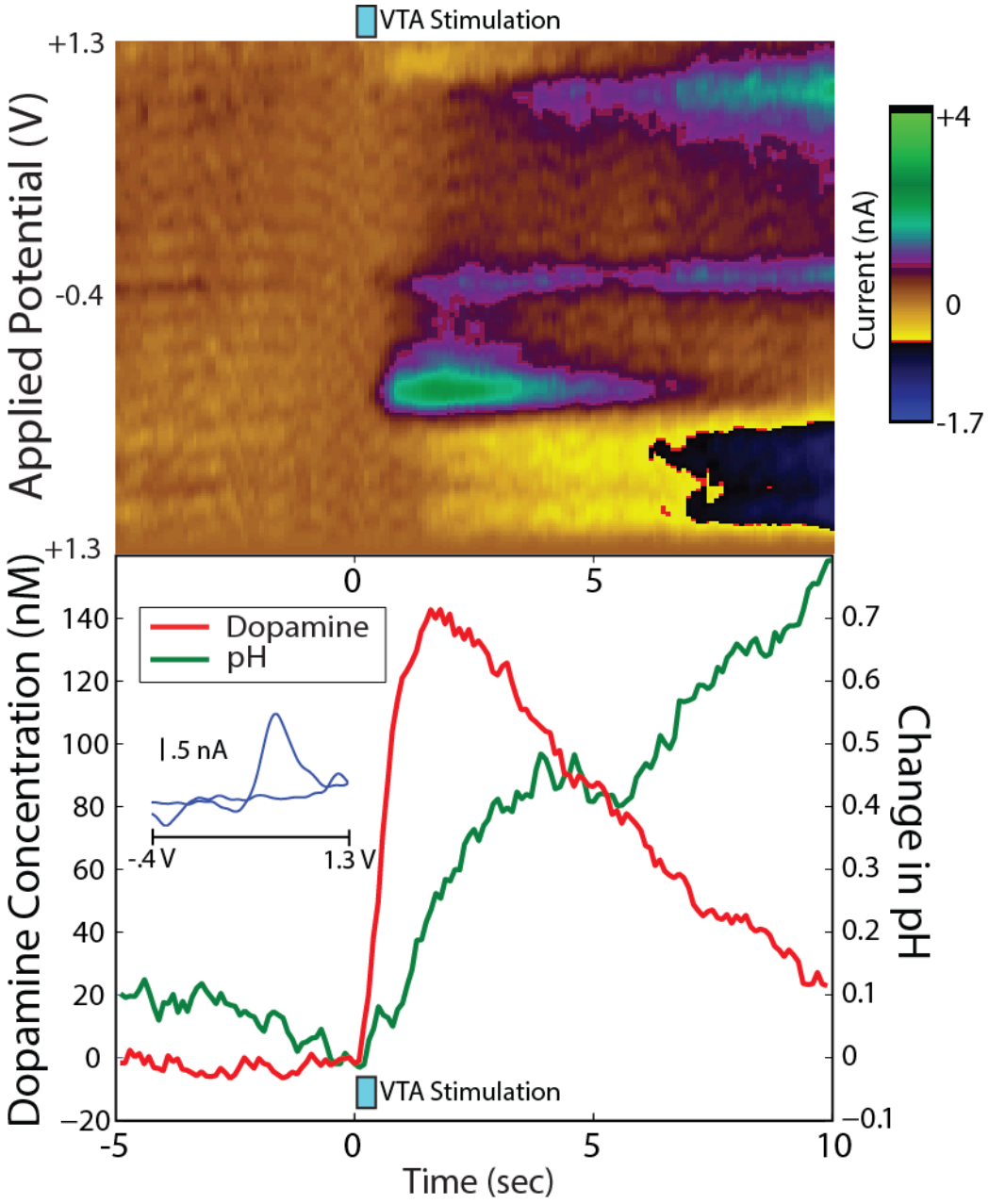

Figure 2.3. DA release evoked by stimulation of the VTA in a behaving animal

A representative pseudocolor plot (top) showing oxidation of DA (purple-green in pseudocolor) just after the onset of electrical stimulation of the VTA (120 μA, 60 Hz, 24 pulses; t = 0; blue rectangle). Graph of calculated DA release (red) and pH shift (green) from the same trial (bottom) with electrical stimulus onset indicated (t = 0; blue rectangle). The oxidation peak occurs 0.67 V, as seen in the cyclic voltammogram (inset).

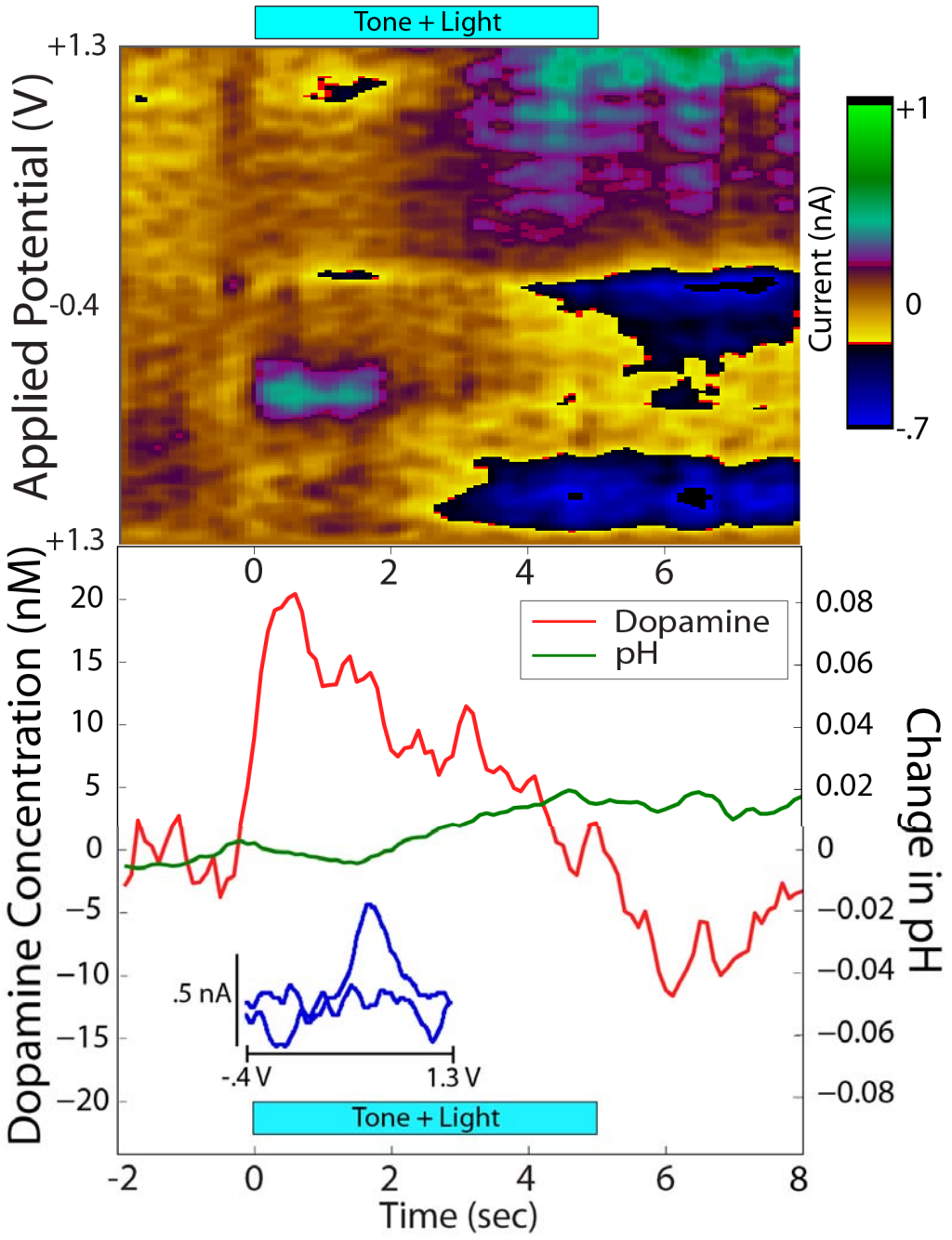

Figure 2.4. DA release evoked on the onset of sucrose-predictive cues

A representative pseudocolor plot (top) showing oxidation of DA (purple-green in pseudocolor) just after the presentation of the predictive cue (t = 0; blue rectangle). Graph of calculated DA release (red) and pH shift (green) from the same trial (bottom) with stimulus onset indicated (t = 0; blue rectangle). The oxidation peak occurs .65 V, as seen in the cyclic voltammogram (inset).

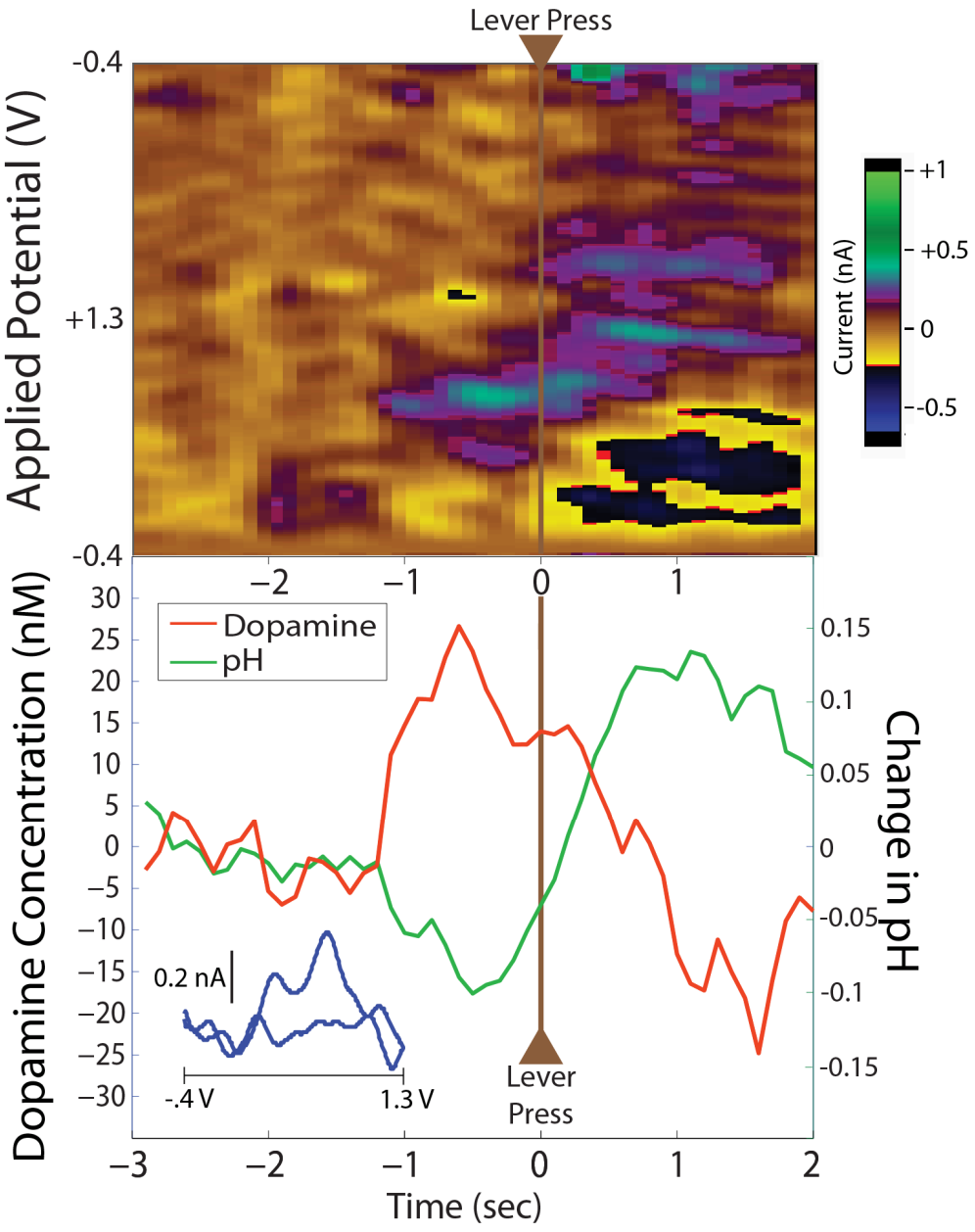

Figure 2.5. DA release evoked by lever press for ethanol delivery

A representative pseudocolor plot (top) showing oxidation of DA (purple-green in pseudocolor) just after the presentation of the predictive cue (t = 0; brown line and arrow). Graph of calculated DA release (red) and pH shift (green) from the same trial (bottom) with stimulus onset indicated (t = 0; brown line and arrow). The oxidation peak occurs 0.70 V, as seen in the cyclic voltammogram (inset).

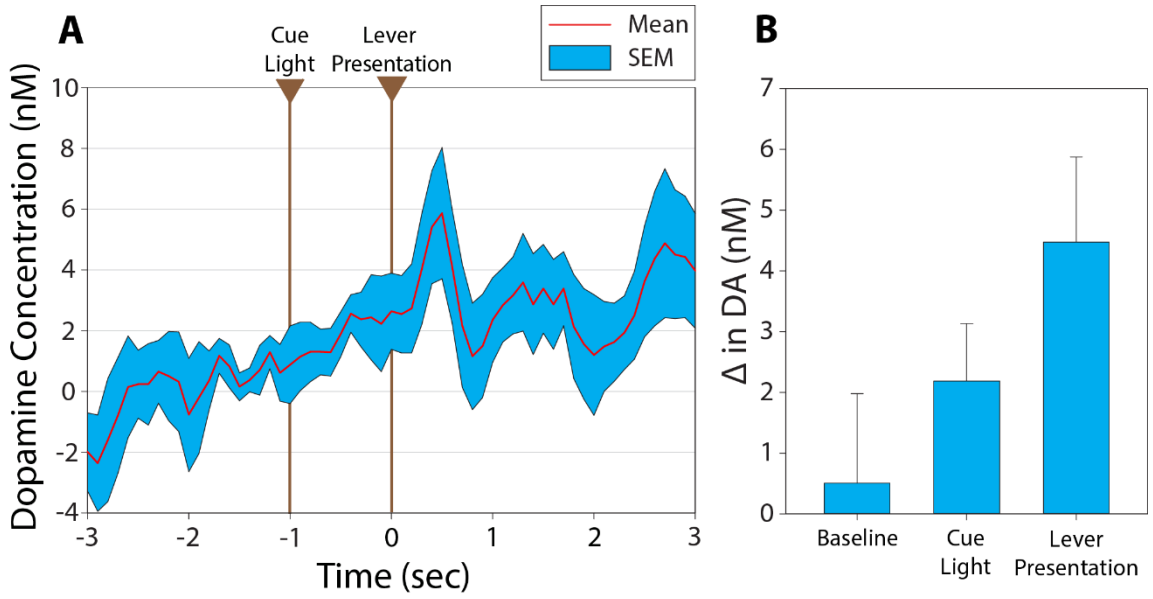

Figure 2.6. DA release is not evoked upon cues predictive of ethanol delivery in food-deprived animals

A, DA concentrations during the presentation of cues that predict ethanol availability (cue light and lever press; n = 4 rats). ***B***, Change in DA concentration after cue presentation for both cues was not significantly elevated relative to the change in baseline DA.

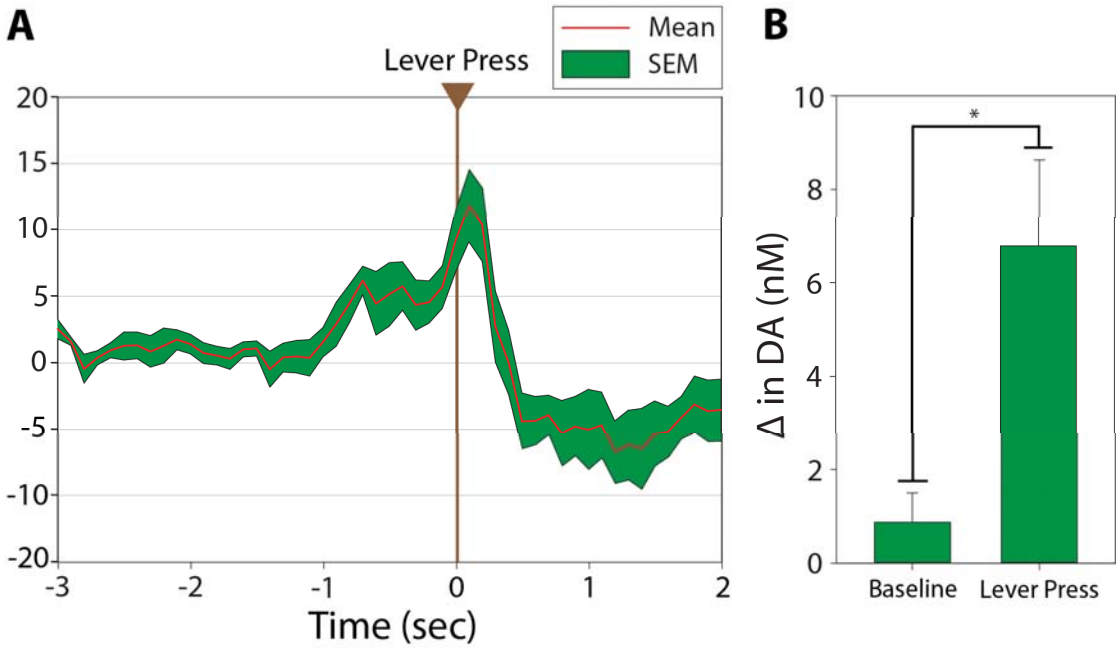

Figure 2.7. DA release evoked for ethanol-rewarded lever press in food-deprived animals

A, DA concentrations during lever pressing for ethanol delivery (n = 5 rats). ***B***, Change in DA concentration after cue presentation was significantly elevated relative to the change in baseline DA (* $p < .05$).

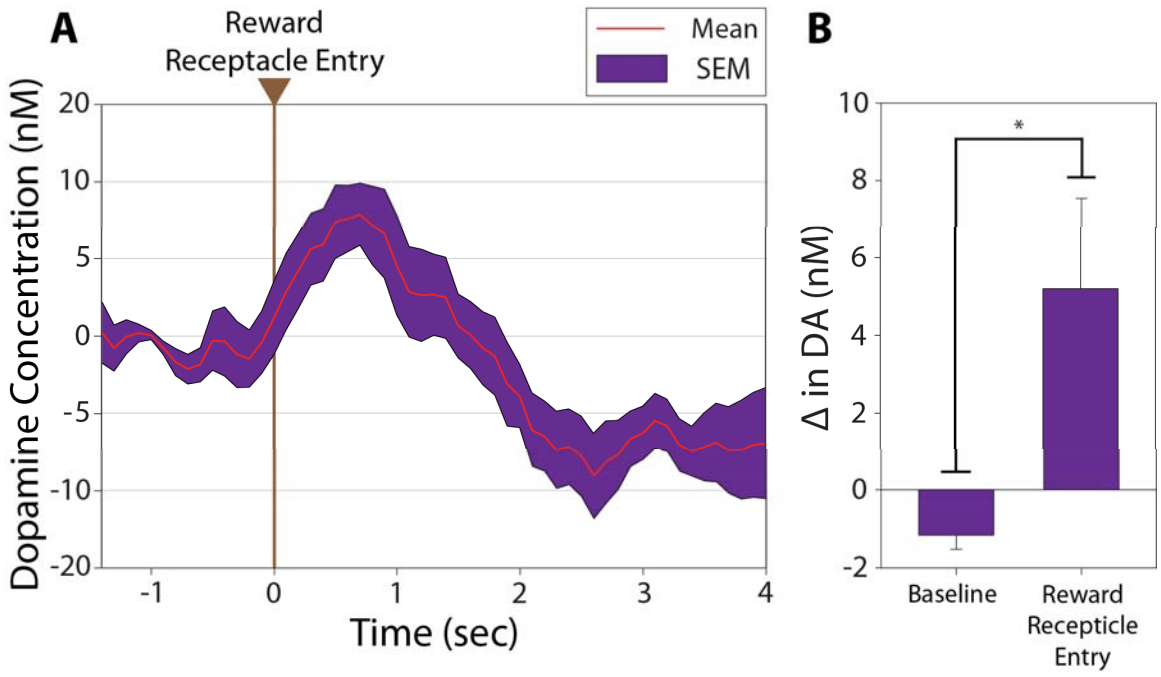

Figure 2.8. DA release is evoked during ethanol consumption in food-deprived rats

A, DA concentrations during ethanol consumption (n = 5 rats). ***B***, Change in DA concentration during ethanol consumption was significantly elevated relative to change in baseline DA (* $p < .05$).

CHAPTER 3

CHARACTERIZATION OF SUBSECOND DOPAMINE RELEASE IN THE NUCLEUS ACCUMBENS CORE DURING OPERANT ETHANOL SELF-ADMINISTRATION

Abstract

Substantial evidence suggests that release of the neurotransmitter dopamine in the nucleus accumbens is crucial for reward-seeking behavior. However, the contribution of subsecond, phasic dopamine release to ethanol-seeking behavior remains poorly understood. Microdialysis studies have shown that dopamine release occurs during anticipatory and consummatory periods of ethanol seeking, but it remains unclear which specific components of ethanol seeking are associated with phasic dopamine signaling in the nucleus accumbens core. Rats were trained in operant self-administration of 20% ethanol using a two-stage training paradigm. Voluntary intake of 20% ethanol escalated during an initial period of intermittent ethanol access in the home cage. Subsequently, animals were trained in an FR1 paradigm to lever press for delivery of 20% ethanol. After rats reached criterion levels of performance, fast scan cyclic voltammetry was used to study phasic DA release in the nucleus accumbens core during operant ethanol

self-administration sessions. Fast scan cyclic voltammetry showed that dopamine levels transiently increased during two behavioral epochs during ethanol seeking. First, dopamine levels increased just after presentation of a cue predictive of ethanol delivery. In addition, dopamine levels increased prior to and peaked shortly after lever press. While some phases of ethanol-seeking have been established to evoke dopamine release, these results demonstrate that dopamine release is evoked by specific components of the behavior. Furthermore, they are consistent with previous studies of dynamic dopamine modulation during food- and cocaine-seeking and suggest a general role for phasic dopamine release in facilitating cue-driven learning and/or behavior, and in motivating reward-seeking operant behavior.

Introduction

Characterizing neural mechanisms underlying ethanol-seeking behaviors is critical to understanding excess ethanol consumption that occurs in alcoholism. Convergent evidence suggests that phasic dopamine (DA) increases in the nucleus accumbens (NAcc) are crucial for reward-seeking behavior. However, the role of DA signaling during ethanol-seeking remains poorly understood. Studies using pharmacological manipulations to investigate the role of DA transmission during ethanol-seeking in rats have yielded mixed results. Increasing DA transmission in the NAcc through infusion of the indirect DA agonist amphetamine increases ethanol-seeking (Samson et al., 1999), and conversely, reducing DA transmission through infusion of DA antagonists attenuates ethanol-seeking

(Czachowski et al., 2001). However, these results run contrary to reports that DA antagonists infused into the NAcc increase (Levy et al., 1991) or that systemic injections have no effect on ethanol-seeking (Goodwin et al., 1996). Furthermore, 6-hydroxydopamine lesions of the NAcc have no effect on ethanol consumption (Ikemoto et al., 1997) or operant ethanol-seeking (Rassnick et al., 1993). Thus, the functional role of DA in mediating ethanol-seeking remains incompletely understood.

In addition, it remains unclear which specific stimulus or behavioral event evokes DA release during operant self-administration of ethanol. Ethanol has been shown to increase firing rates through direct action on dissociated DA neurons of the VTA (Brodie et al., 1999b). Microdialysis studies have shown that both oral consumption (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002) and intraperitoneal injection (Imperato and Di Chiara, 1986) result in a gradual increase followed by prolonged elevation of striatal DA, a time course consistent with a pharmacological mechanism for ethanol-evoked DA release. FSCV studies have also shown that injection of moderate to high ethanol doses pharmacologically potentiate DA release in the NAcc (Cheer et al., 2007; Robinson et al., 2009). These results suggest the pharmacological effects of ethanol drive DA release in the striatum.

However, conflicting results from microdialysis studies show that increased DA levels after self-administration is transient relative to the much longer period in which blood ethanol concentrations (BECs) rise (Doyon et al., 2003, 2005). These results suggest that increases in DA during self-administration occur

independently of ethanol's pharmacological effects and implicate aspects of ethanol-seeking behavior in evoking DA release. Specifically, DA levels in the NAcc have been reported to increase during an anticipatory period prior to ethanol self-administration (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002). However, subsequent work has suggested that DA release during this anticipatory period may be caused by handling, and therefore is not specifically related to anticipation of ethanol (Doyon et al., 2003).

Several studies have used microdialysis to characterize DA release during operant ethanol-seeking and their recordings reveal robust DA release during this behavior (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002). Yet, in these studies lever pressing and ethanol consumption occurred during the same reporting period, making it unclear if DA release is evoked for lever press or consumption. A subsequent microdialysis study temporally separated periods of instrumental responding from ethanol consumption behaviors (Doyon et al., 2003). In this report, ethanol consumption resulted in increased DA levels but lever pressing was not found to evoke DA release. Recently, an increase in phasic DA was recorded during lever press in the dorsolateral striatum (DLS) and NAcc for receipt of a sweetened ethanol solution (10% sucrose and 10% ethanol; Shnitko and Robinson 2014). While this report found increased phasic DA in the DLS during ethanol-seeking, increases recorded in the NAcc were combined between animals responding for sweetened ethanol and 10% sucrose solution. Therefore, it remains unclear if DA release is evoked during an ethanol-rewarded lever press.

In the present study, I used FSCV to investigate how ethanol-predictive

cues, ethanol-seeking behaviors and the pharmacological effect of ethanol affect phasic DA release in the NAcc. I took advantage of the high temporal resolution provided by the FSCV technique to examine DA release on a behaviorally-relevant timescale and identify specific task events associated with DA release. I found that phasic DA release accompanied delivery of cues predictive of ethanol delivery and operant ethanol-seeking behavior. Moreover, the magnitude of DA release evoked by ethanol predictive cues was found to be correlated to lever press latency. These results suggest a role for phasic DA in mediating ethanol-directed operant responding.

Methods

Animals

Male Wistar rats (175-225 g, Charles River) were used in all experiments. Animals were individually housed and had *ad lib* access to food and water throughout the experiment. Six rats were given intermittent ethanol access (IEA) in the home cage, and then trained in operant self-administration of ethanols (see details below). These rats were then implanted with FSCV electrodes for DA detection. In a separate group of 16 unimplanted rats, BECs were measured after operant responding for ethanol. All procedures used were in accordance with the Institutional Animal Care and Use Committee at the University of Utah and in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Overview of experimental design for FSCV

Experiments were conducted in three phases. Rats were initially trained to consume ethanol in home cages using an IEA paradigm. Rats in which home cage drinking levels reached criterion levels (see details below), were then trained to self-administer ethanol in an operant self-administration paradigm. Finally, phasic DA in the NAcc was recorded during ethanol self-administration.

Home cage ethanol consumption

Training to consume ethanol occurred using an initial period of IEA in the home cage, followed by operant training (Simms et al., 2010). Upon receipt into the animal facility, rats were individually housed and allowed 1 week to acclimate to their surroundings. Rats were then given 24 hour access to 20% ethanol (v/v) and water in their home cage 3 days per week (Monday, Wednesday and Friday). Ethanol and water bottle positions were alternated on successive ethanol access days to avoid the development of a side preference. Rats reaching a criterion level of ethanol consumption (≥ 3 g/kg/24 hours) for 3 consecutive drinking sessions were implanted with voltammetry electrodes directed to the NAcc core. This threshold was chosen because my previous work showed that rats meeting this criterion were more likely to reach pharmacologically relevant BECs (≥50 mg/dl; Haack et al. 2014). IEA lasted between 2-7 weeks prior to voltammetry electrode implantation to ensure all rats reached the criterion level of ethanol consumption in home cage. After electrode implantation, rats were provided an additional 3 weeks IEA access prior to operant self-administration training. Total IEA thus

lasted for 5-10 weeks (mean = 7.0 ± 1.0 weeks).

Stereotaxic FSCV electrode implantation

Rats were induced and maintained using isoflurane anesthesia (5% and 2% in O_2, respectively) for stereotactic implantation of FSCV carbon fiber electrodes. After placement in the stereotax, the head was shaved and the scalp was swabbed with ethanol solution followed by betadine (10%, Purdue Frederick, Stamford, CT). An incision was made to expose the cranium. Using a dental drill, a burr hole was drilled into the cranium to target the NAcc core (AP: 1.3, ML: 1.3, DV: -7.2). A carbon fiber electrode that was fabricated in-house was lowered into the NAcc and an Ag/AgCl electrode was positioned into a region of contralateral forebrain. All electrodes were encased in cranioplastic cement and secured to the skull with 4 stainless steel screws. After surgery, buprenorphine (0.1 mg/kg IP, Reckitt Benckiser, Richmond, VA) and penicillin (6000 U/kg IM, VetOne, Boise, ID) were administered for analgesia and to prevent infection, respectively. Rats were allowed a 1 week recovery period after implantation before resumption of operant ethanol self-administration training.

Operant ethanol self-administration training

After recovery from surgery, rats were given an additional 3 weeks of IEA reminder sessions. Next, implanted rats were trained to lever press for ethanol in operant test chambers (Med-Associates, St. Albans, VT). Operant chambers contained a reward receptacle flanked by two retractable levers with cue lights

above the levers and were used exclusively for training prior to FSCV voltammetry recording, which took place in a separate operant chamber. Rats were first given overnight (14 hour) training sessions on alternate days until reaching a criterion of 40 lever presses in a single overnight session. For most rats, this occurred within a few sessions (mean = 1.5 ± 0.3 sessions).

After meeting the criterion number of lever presses, rats were trained to perform the task overnight in the voltammetry testing chamber. This chamber was similar in design to the training chambers, and included a central reward receptacle flanked by retractable levers. During this session, the electrode was connected to a commutator to habituate the rat to operant responding while tethered, but DA levels were not recorded. After meeting a response criterion of 40 lever presses in an overnight session, DA levels were recorded during a 2 hour interval of operant ethanol self-administration in the voltammetry testing chamber. A single recording session was performed for each implanted rat.

Operant ethanol self-administration

In the operant paradigm, each trial was initiated by simultaneous illumination of a cue light and extension of a single lever into the cage to signify ethanol availability. After an operant response, the cue light extinguished, a 1 second delay followed, and then ethanol (0.1 mL of 20% ethanol) was delivered into the reward receptacle. Ethanol delivery was accompanied by house light illumination and presentation of a 1 kHz tone (5 second duration). A randomized 10-30 second intertrial interval (mean = 20 seconds ± 3.5) preceded initiation of

the next trial.

Voltammetric measurements and analysis

Carbon fiber microsensors were used to detect DA concentration in the NAcc (Clark et al. 2010). Chronic electrodes were used in combination with a compact head stage, allowing free movement of the rat and reducing stress when tethering the rat prior to recording. A custom built, head mountable amplifier for voltammetric signals was connected to the carbon fiber electrodes before recording. Free movement of the rat in the test chamber was ensured by routing the cable from the amplifier through an electrical commutator (Crist Instruments, Hagerstown, MD). Generation of the voltammetric waveform and data collection was performed with custom software coded in LabVIEW for PC-based data acquisition hardware (National Instruments, Austin, TX).

Prior to recording DA during ethanol self-administration, rats were placed in the test chamber and the voltammetric scan was cycled at a rate of 60 Hz to equilibrate the carbon fiber electrode. Thereafter, DA levels in the NAcc were recorded during the operant ethanol self-administration task described above. During operant self-administration, recorded current was background subtracted from a 10 scan average taken at the beginning of each experimental trial. Signals were band-pass filtered (0.025-2000 Hz) and smoothed by taking a 5 scan running average of cyclic voltammograms. Chemometric analysis was used to isolate DA signal from other analytes (Heien et al., 2004). To entrain the chemometric analysis, a training set was used that contained electrically-evoked signal of DA

release, pH shift and electrode drift (Clark et al., 2010).

Statistical analysis

Task related changes in DA concentrations were analyzed in response to cue presentation (light illumination and extension of the operant response lever) and during operant responding for ethanol (lever pressing). DA concentrations were analyzed in 500 ms bins. Significant changes in DA concentration after task events were identified by comparing DA concentrations in the 500 ms bin after the cue/operant response to baseline DA concentrations (paired t-test). In order to determine the relationship between phasic DA release evoked for cue presentation and lever press latency, a correlation was performed. For correlation analysis, mean DA release evoked in the 500 ms after cue presentation or lever press was subtracted from a 1 second period of baseline DA at the beginning of the trial to calculate change in DA from baseline. The relationship between the timing of behavioral events was determined by comparing to the change in DA using a Pearson product-moment correlation. All statistical analysis was performed using SPSS software (IBM, Armonk, NY).

Verification of recording sites

Rats were deeply anesthetized with a cocktail of pentobarbital and phenytoin (Beuthanasia, 390 mg pentobarbital 50 mg phenytoin per mL, Schering-Plough, Kenilworth, NJ). Recording sites were marked by creating an electrolytic lesion (300 V) prior to intracardiac perfusion with saline followed by 4% formalin.

Brains were extracted, postfixed and cryoprotected with 30% sucrose. Tissue sections were sliced in coronal sections at 50 µm with a freezing sliding microtome (Leica, Buffalo Grove, IL). Once mounted, sections were stained with cresyl violet and electrode locations were verified to be in the NAcc core as per Paxinos and Watson (2007).

Blood ethanol concentration (BEC) determination

To determine BECs achieved after operant ethanol self-administration, BECs were measured in a separate group of unimplanted rats. Nineteen rats were trained to consume ethanol in the IEA paradigm for 5 weeks and trained on the operant paradigm as described previously. Of these, 16 consumed ethanol in excess of 3 g/kg/24 hr and advanced to operant training. This was carried out as described above in *Operant ethanol self-administration training.* BECs were measured after a single 30 minute testing session for a more accurate determination of the peak BEC experienced during the task. Once rats had completed 30 minutes in the operant session, they were anesthetized with isoflurane and tail vein blood was collected into heparinized capillary tubes. Ethanol concentrations were measured using the NAD-NADH spectrophotometric assay (Zapata et al., 2006).

Results

Behavior

Rats were first trained to consume ethanol in their home cages using an intermittent ethanol access (IEA) paradigm. In this paradigm, rats had access to 20% ethanol 3 days per week on alternating days for at least 5 weeks (water and food were always available *ad libitum)*. Exposure to the IEA paradigm resulted in escalation of voluntary consumption of 20% ethanol (mean intake = 6.0 ± 0.5 g/kg/24 hr during the 5th week of training, Figure 3.1a). After IEA exposure, rats were transitioned to operant ethanol self-administration training. All rats met a criterion of 40 responses during overnight sessions during the final training session prior to FSCV recording (mean number of lever presses = 87.2 ± 11.9, Figure 3.1b).

Cue-evoked dopamine release

Phasic DA was recorded during operant self-administration sessions in which each lever press resulted in ethanol delivery (Figure 3.1c). All recording electrodes were confirmed to be localized to the NAcc cord (Figure 3.2).

During 2-hour FSCV recording sessions, rats averaged 11.3 ± 2.6 lever presses. Presentation of a cue (lever extension + cue light) that predicted ethanol availability resulted in a transient increase in DA concentration occurring at short latency after cue presentation (Figure 3.3). In a representative trial, DA concentration (purple/green signal in psuedocolor heat plot, Figure 3.3a), increased just after the presentation of the predictive cue and peaked

approximately 300 ms after cue presentation. Consistent with the electrochemical signature of DA, oxidization of the analyte occurred near 0.60 V, as highlighted in the cyclic voltammogram (Figure 3.3b, inset). Increased DA release evoked by ethanol predictive cues was robust, as reflected in the mean DA change after cue presentation (Figure 3.3c). Peak DA release occurred an average of 400 ms after cue presentation and DA in all rats had peaked an average of 600 ms after cue presentation. Signal declined to baseline levels within 1 second after cue presentation. The increase in DA concentration occurring after cue presentation was significant relative to baseline DA concentration (Figure 3.3d, $p < 0.05$).

In most behavioral trials, rats' lever pressing occurred shortly after cue presentation. This raised the question of whether cue-evoked DA release increases were in fact dependent on the operant response following the cue, rather than the cue itself. To address this question, I analyzed the timing of lever pressing relative to cue presentation. In trials less than 60 seconds, the latency to lever press occurred long after cue-evoked DA signal peaked (Figure 3.4, median = 8.7 seconds). In only a small minority of trials (3%) was the latency to lever press less than 1 second, the temporal window over which cue-evoked signal persisted. The substantial temporal lag between cue-evoked DA release and the subsequent operant response thus suggests that the early increase in DA concentration was associated with cue presentation itself, rather than the lever press.

Multiple lines of evidence suggest that cue-evoked DA signals contribute to subsequent operant responding (Berridge and Robinson, 1998; Wassum et al., 2012a). To determine if the magnitude of cue-evoked DA release was related to

the timing of subsequent lever pressing, lever press latencies following cue presentation were calculated for each trial and compared to the magnitude of cue-evoked DA increases. The magnitude of DA signal and latency to respond were indeed correlated, with a significant negative relationship of cue evoked DA release to lever press latency (Figure 3.5, $p < .01$, $r = -.334$). These data are consistent with a role for cue-evoked DA release in driving subsequent operant responding.

Dopamine release during operant responding

DA release was consistently associated with lever pressing for ethanol reward (Figure 3.5). In the representative pseudocolor plot (Figure 3.5a) and graph showing DA concentration (Figure 3.5b), DA levels increased before the lever press and peaked roughly 100 ms after the lever press. Oxidative current occurred at an applied voltage of 0.66 V, as seen in the cyclic voltammogram (Figure 3.6b, inset) and in the pseudocolor plot, consistent with DA signal. DA concentrations peaked at an average of 200 ms after lever press (Figure 3.6c). The increase in DA occurring after the lever press was significant relative to baseline DA concentrations (Figure 3.6d, $p < 0.01$). Unlike cue-evoked DA release, the magnitude of lever press evoked DA release was not correlated with latency to lever press (data not shown, $r = -.05$, $p = 0.69$).

Phasic DA release is unrelated to levels of ethanol intake

To determine if the pharmacological effects of ethanol contributed to phasic DA signals measured in the current study, I analyzed the relationship between operant response levels and phasic DA increases in each rat. There was no relationship between the number of trials completed by each rat and phasic DA signal evoked by cue- (data not shown, $r = -.74$, $p = 0.09$) or lever press-evoked DA release (data not shown, $r = .06$, $p = 0.91$). In order to confirm that the number of operant responses completed were correlated with BEC, I analyzed the relationship between these two variables in a separate group of animals that were not implanted for voltammetry (n = 16 rats). These animals were given the same sequence of IEA and operant training as rats used in the voltammetry study. The number of lever presses completed was highly correlated with BEC (Figure 3.7, $r = .927$, $p < 0.001$). While BECs were not measured in rats in which FSCV was performed, these animals averaged 11.3 ± 2.6 lever presses during recording sessions, a response rate that would be expected to produce BECs of approximately 27.9 mg/dl.

Discussion

DA signaling in the NAcc is known to play a central role in reward seeking. However, our understanding of DA signaling during ethanol self-administration remains incomplete. Indeed, the temporal dynamics of DA release accompanying operant ethanol self-administration have received little attention, and previous studies are in conflict regarding the specific task or behavioral events associated

with DA release. In this study, I took advantage of the high temporal resolution provided by FSCV to explore the role of DA during operant ethanol self-administration. I found that both ethanol-predictive cues and operant responding itself are associated with phasic increases in DA concentration. Potential roles of these signals in ethanol-seeking are discussed in further detail below.

My results show that presentation of ethanol-predictive cues is associated with a transient increase in DA concentration. Moreover, I found that the magnitude of this cue-evoked DA release was inversely correlated with the latency to initiate an operant response, with the largest increases in DA concentrations occurring on trials in which response latency was the shortest.

DA signaling has been hypothesized to serve a number of distinct roles in driving motivated behavior. My findings are consistent with the proposal that cue-evoked DA signaling serves an important role in driving behavioral responding prompted by presentation of cues that signal reward availability (Berridge and Robinson, 1998). Consistent with this interpretation, infusion of DA receptor antagonists into the NAcc delays the initiation of approach behavior to a rewarded-associated lever (Nicola, 2010) and increased phasic DA is predictive of more rapid completion of a sequential food-seeking task (Wassum et al., 2012a). My results indicate that DA release evoked by ethanol-predictive cues may similarly serve to accelerate ethanol-seeking behaviors.

Notably, I found that DA release in the NAcc occurred not only after cues predicting reward availability, but also accompanied the operant response itself. I observed a significant increase in DA concentrations that ramped up prior to and

peaked just after the occurrence of an operant response. Studies of rats engaged in cocaine- (Phillips et al., 2003b) and food-seeking (Roitman et al., 2004) have identified comparable increases in subsecond DA during lever pressing for these rewards. This similarity suggests a similar dopaminergic mechanism mediates responding for varying types of reinforcement.

A recent study examined phasic DA levels in the dorsal striatum and NAcc during self-administration of a sweetened ethanol solution (Shnitko and Robinson, 2014). This study importantly differs from my own in using sucrose to increase motivation for the ethanol reward. The authors reported that lever press-associated DA increases occurred in both the DLS and NAcc during operant self-administration, and that the magnitude of this increase was nearly identical in rats responding for sweetened ethanol (10% sucrose + 10% ethanol) and in those responding for a pure sucrose (10% sucrose) solution. However, instrumental responding for sucrose is well known to lead to robust phasic DA (Roitman et al., 2004), leaving open the specific role of ethanol in eliciting phasic DA responses. Cue-evoked DA responses were not explicitly characterized in this study. My results are thus novel in demonstrating that ethanol-predictive cues and operant responding during ethanol-seeking behavior is associated with the release of phasic DA in the NAcc.

The pharmacological effect of ethanol has been shown to increase the activity of VTA dopaminergic neurons *in vitro* (Brodie et al., 1999b), and *in vivo* microdialysis studies have recorded a dose-dependent increase in NAcc DA levels upon operant ethanol self-administration (Gonzales and Weiss, 1998). These

observations raise the possibility that the pharmacological effects of ethanol contribute to DA release during operant self-administration. In the present study, however, I found no relationship between ethanol consumption and phasic DA release. These results are consistent with previous microdialysis studies showing that increased DA concentration occurring during ethanol self-administration increases with a time course that is dissociated from the time course of ethanol concentration in the brain (Doyon et al., 2003, 2005). While I found no relationship between level of operant self-administration and phasic DA magnitude (for either cue or lever press-evoked release), I cannot rule out the possibility that higher levels of ethanol intake than those reached in my study would increase phasic DA signals.

My data show that both ethanol predictive cues and operant ethanol-seeking both evoke subsecond DA release in the NAcc. DA in the NAcc has been suggested to encode the incentive salience for an anticipated reward (Berridge and Robinson, 1998). My finding of DA release evoked by ethanol-predictive cues is consistent with the possibility that phasic DA in the NAcc encodes the value or incentive salience of ethanol. Indeed, I found that DA release evoked by ethanol predictive cues is inversely related to rats' latency to lever press, thereby suggesting cue-evoked DA release speeds operant responding. My demonstration of phasic DA during ethanol-seeking, along with prior reports of similar signal during cocaine- and sucrose-seeking, indicates a general role for phasic DA in motivating reward-seeking behavior.

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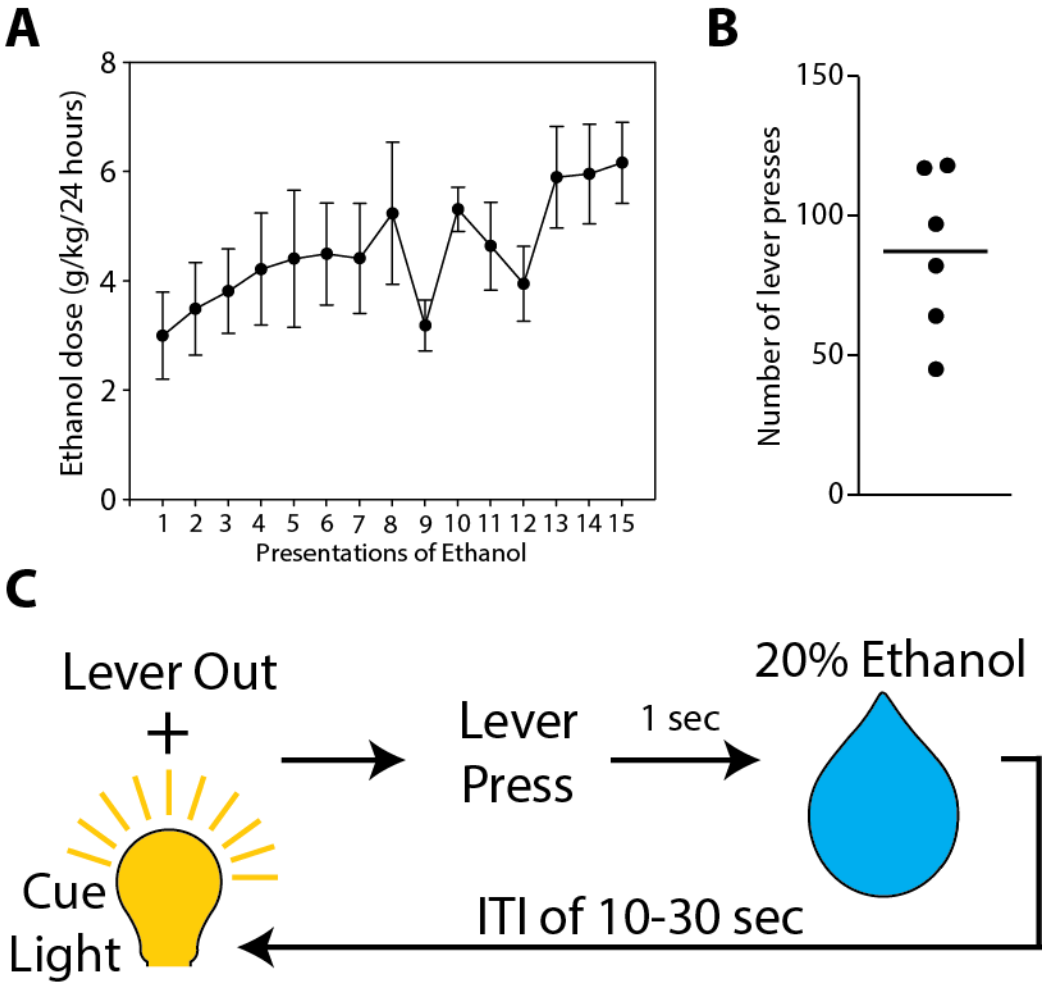

Figure 3.1. Experimental design

A, Escalation of 20% ethanol consumption in home cage in 5 weeks of training. ***B***, Number of lever presses during the final overnight operant ethanol self-administration training session. ***C***, Design of operant ethanol self-administration paradigm, with cue presentation (lever out and cue light), followed by lever press and delivery of 20% ethanol. A randomized intertrial interval (ITI) followed each successful trial completion.

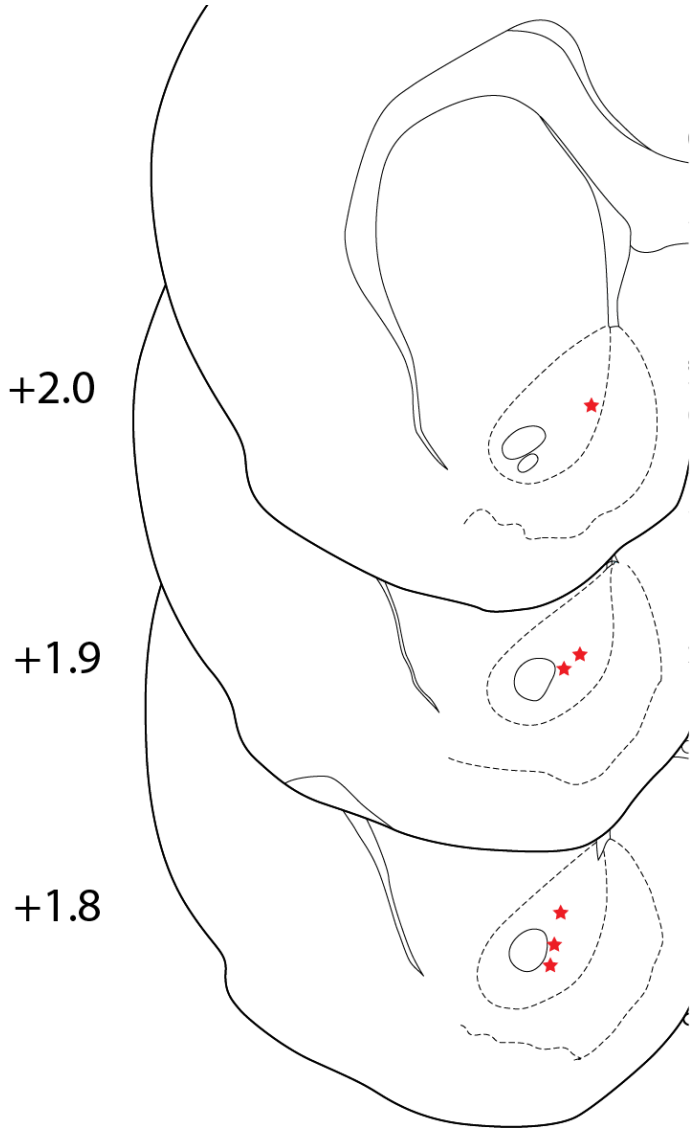

Figure 3.2. Electrode locations

Locations of implanted carbon fiber electrodes. All placements were confined to the NAcc core.

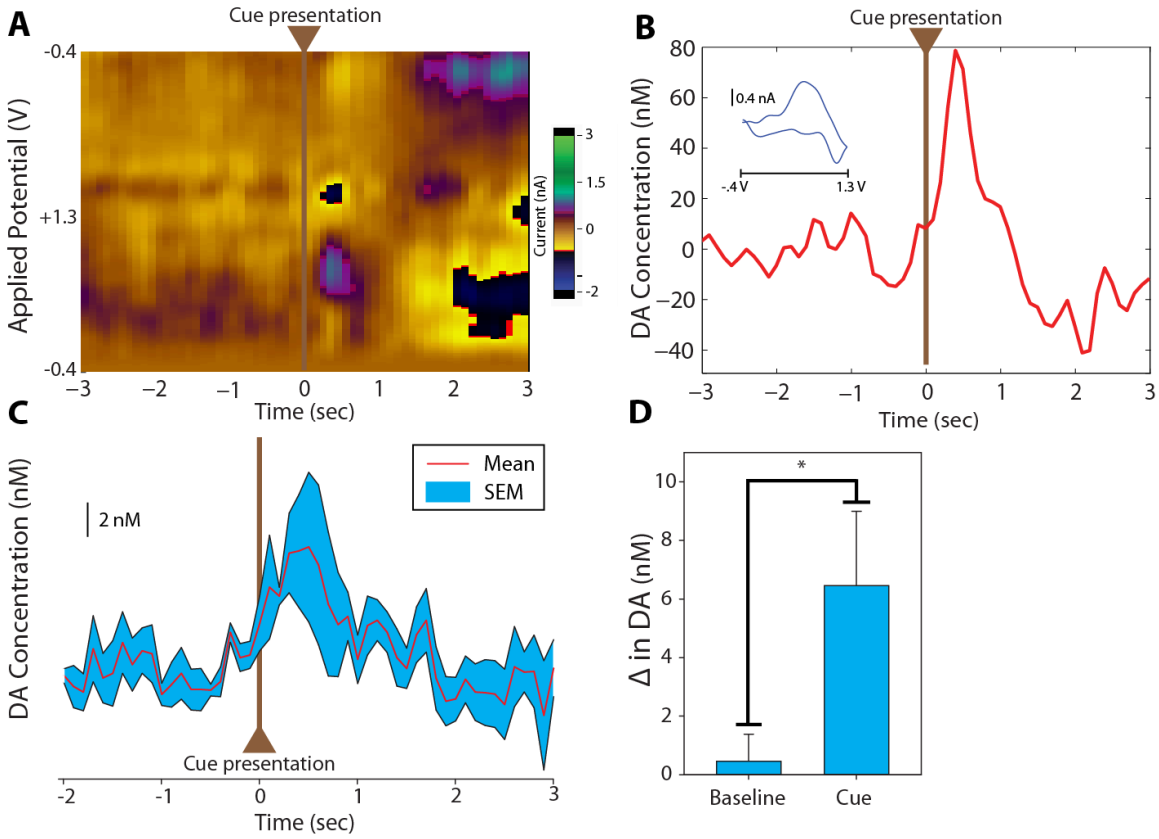

Figure 3.3. DA release evoked by ethanol predictive cues

A, A representative pseudocolor plot showing oxidation of DA (purple-green in pseudocolor) just after the presentation of the predictive cue (brown line and triangle). ***B***, Graph of calculated DA release from the same trial. The oxidation peak occurs at 0.60 V, as seen in the cyclic voltammogram (inset). ***C***, Graph of mean DA release (n = 6 rats) evoked and SEM (shaded) from the presentation of cues (t = 0, brown line and triangle) that are predictive of ethanol. ***D***, Change in DA concentration after cue presentation was significantly elevated relative to the change in baseline DA (* $p < .05$).

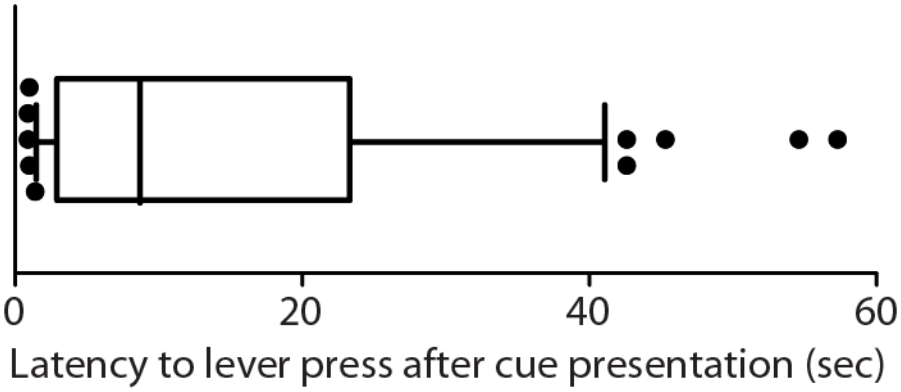

Figure 3.4. Lever press latencies

Cue evoked DA release is independent of lever press evoked DA release. Latency to lever press after cue presentation in rats that lever pressed in less than 60 seconds. Box plot indicates 10^{th}, 25^{th}, median, 75^{th} and 90^{th} percentile values, while circles indicate outliers.

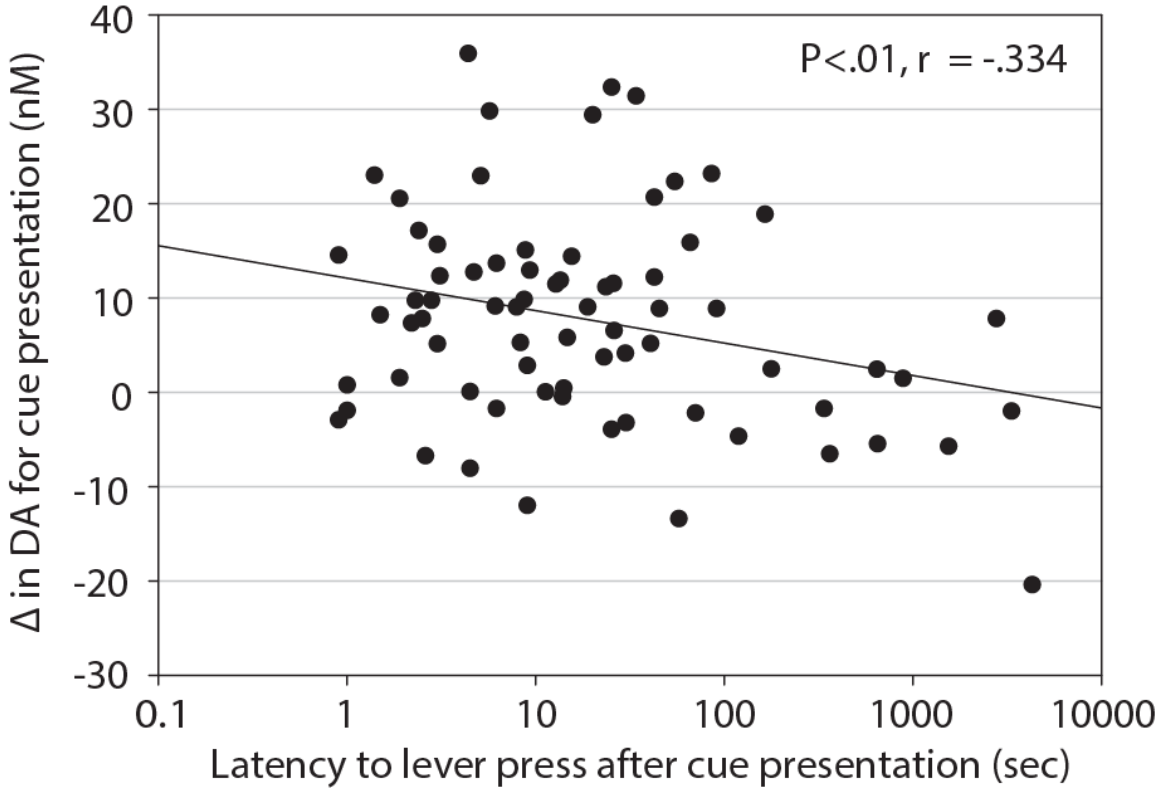

Figure 3.5. Cue evoked DA release is correlated with latency to lever press for ethanol

The change in DA concentration upon cue presentation (y axis) is graphed against the time between cue presentation and latency to lever press after cue presentation (x axis, log scale) for each trial ($p < .01$, $r = -.334$).

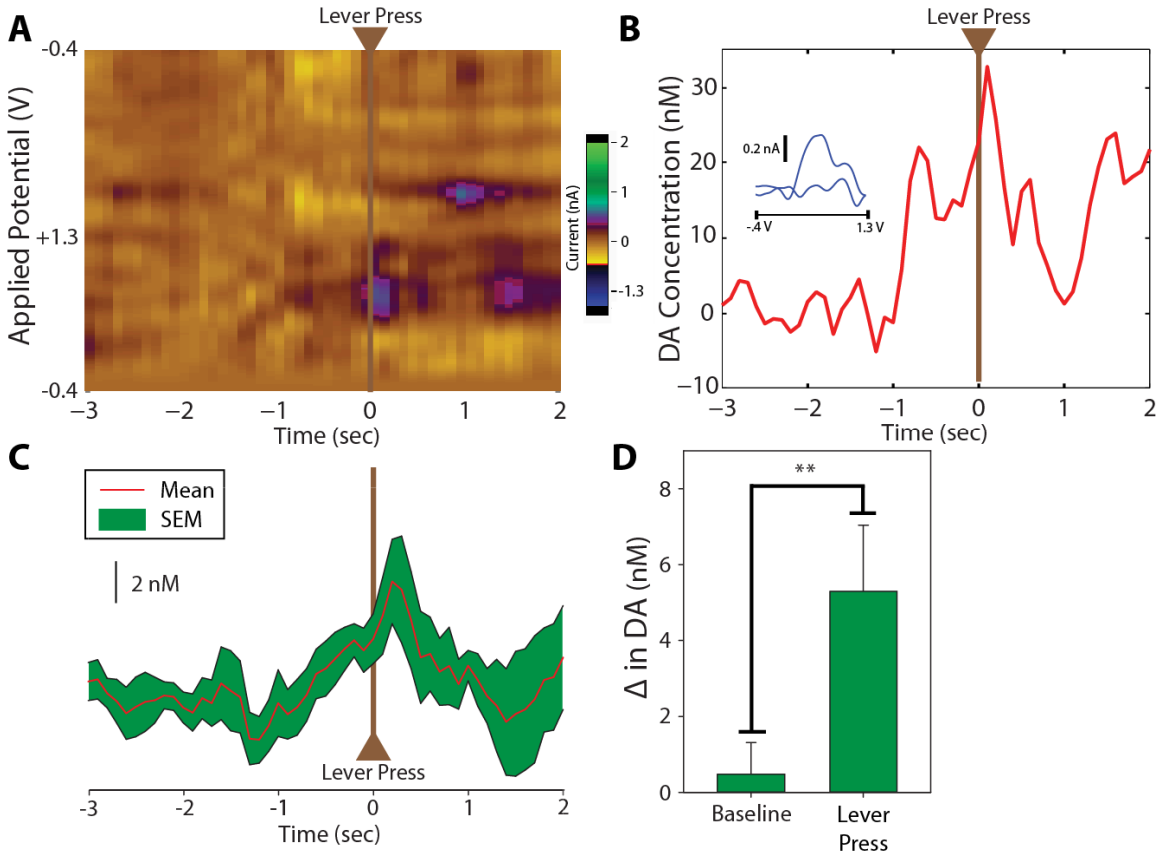

Figure 3.6. DA is released for an ethanol rewarded lever press

A, A representative pseudocolor plot showing oxidation of DA just after lever press (brown line and triangle). ***B***, Graph of calculated DA release from the same trial. The oxidation peak occurs at .66 V, as seen in the cyclic voltammogram (inset). ***C***, Mean DA (n = 6 rats) release evoked at the time of the lever press (t = 0, brown line and triangle) for ethanol. ***D***, Change in DA concentration occurring during the lever press was significantly elevated relative to change in baseline DA (**, $p < .01$).

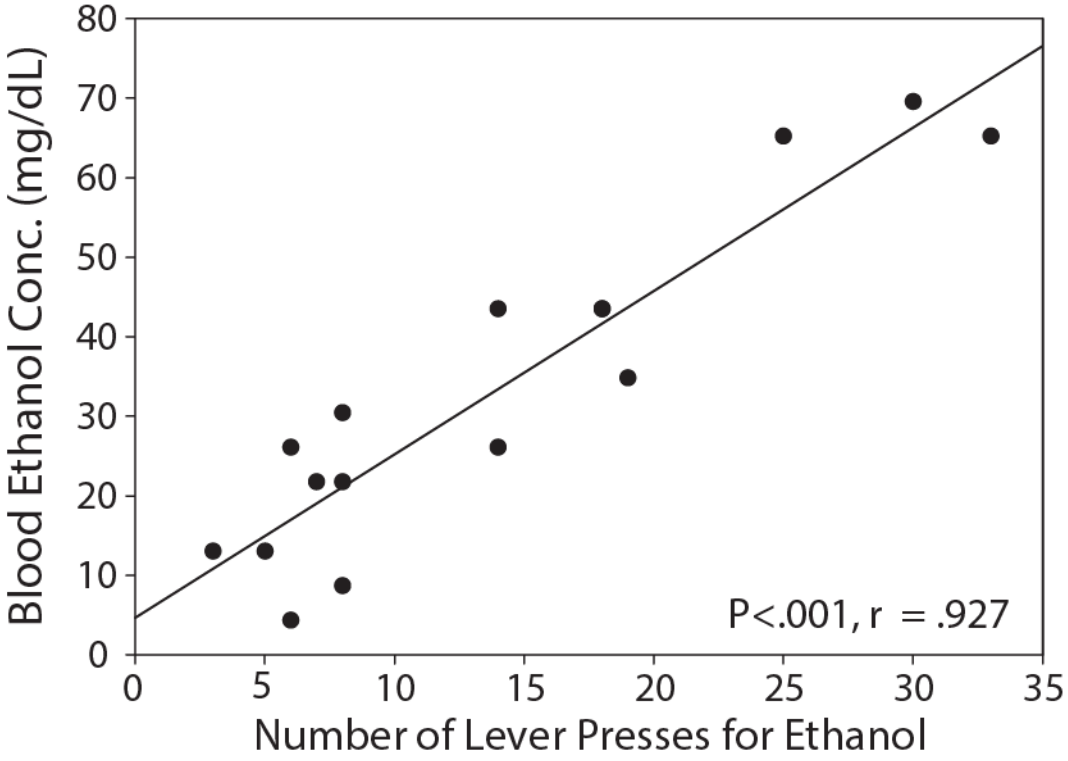

Figure 3.7. Ethanol consumption is correlated to BEC

The number of lever presses occurring during an experimental session was predictive of BEC (n = 15 rats, r = .927, p <.001).

CHAPTER 4

LESIONS OF THE LATERAL HABENULA INCREASE VOLUNTARY ETHANOL CONSUMPTION AND OPERANT SELF-ADMINISTRATION, BLOCK YOHIMBINE-INDUCED REINSTATEMENT OF ETHANOL SEEKING, AND ATTENUATE ETHANOL-INDUCED CONDITIONED TASTE AVERSION

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Lesions of the Lateral Habenula Increase Voluntary Ethanol Consumption and Operant Self-Administration, Block Yohimbine-Induced Reinstatement of Ethanol Seeking, and Attenuate Ethanol-Induced Conditioned Taste Aversion

Andrew K. Haack[1,2☯], Chandni Sheth[3☯], Andrea L. Schwager[1,2], Michael S. Sinclair[1], Shashank Tandon[1], Sharif A. Taha[1,2*], for the Brain Institute

1 Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah, United States of America, **2** Interdepartmental Program in Neuroscience, University of Utah School of Medicine, Salt Lake City, Utah, United States of America, **3** Department of Pharmacology and Toxicology, University of Utah School of Medicine, United States Salt Lake City, Utah, United States of America

Abstract

The lateral habenula (LHb) plays an important role in learning driven by negative outcomes. Many drugs of abuse, including ethanol, have dose-dependent aversive effects that act to limit intake of the drug. However, the role of the LHb in regulating ethanol intake is unknown. In the present study, we compared voluntary ethanol consumption and self-administration, yohimbine-induced reinstatement of ethanol seeking, and ethanol-induced conditioned taste aversion in rats with sham or LHb lesions. In rats given home cage access to 20% ethanol in an intermittent access two bottle choice paradigm, lesioned animals escalated their voluntary ethanol consumption more rapidly than sham-lesioned control animals and maintained higher stable rates of voluntary ethanol intake. Similarly, lesioned animals exhibited higher rates of responding for ethanol in operant self-administration sessions. In addition, LHb lesion blocked yohimbine-induced reinstatement of ethanol seeking after extinction. Finally, LHb lesion significantly attenuated an ethanol-induced conditioned taste aversion. Our results demonstrate an important role for the LHb in multiple facets of ethanol-directed behavior, and further suggest that the LHb may contribute to ethanol-directed behaviors by mediating learning driven by the aversive effects of the drug.

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* E-mail: s.taha@utah.edu

☯ These authors contributed equally to this work.

Introduction

The lateral habenula (LHb) has been importantly implicated in learning driven by adverse outcomes. Neurons in the primate LHb are excited by negative stimuli, such as an aversive air puff or cues predicting the absence of reward [1,2]. Excitation of LHb neurons results in inhibition of dopamine neurons in the ventral tegmental area and substantia nigra pars compacta [3]. This inhibition is mediated by a disynaptic pathway in which excitatory afferents originating in the LHb synapse on GABAergic neurons in the rostromedial tegmental area (RMTg), which target and inhibit downstream dopaminergic neurons [4]. Supporting a role for the LHb-RMTg pathway in learning driven by undesirable outcomes, manipulations that increase firing in the LHb, in LHb efferents to the RMTg, or in the RMTg itself are both acutely aversive and cause aversive conditioning [5–11].

The positively reinforcing effects of drugs of abuse motivate further drug seeking, particularly in initial stages of drug use [12]. However, these drugs also have aversive effects that limit voluntary intake [13]. Recent studies have implicated the habenula in negatively regulating motivation for both nicotine [14] and cocaine [6,15], and provide evidence that habenular circuits mediate learning driven by the aversive effects of these drugs. Ethanol consumption results in aversive effects that include acute sedation and motoric impairment as well as delayed hangover effects [16,17]. Sensitivity to these aversive effects is associated with decreased voluntary ethanol intake in rodent models, as shown by the inverse correlation of ethanol-induced conditioned taste aversion (CTA) with voluntary ethanol consumption and preference [18,19]. Along these lines, increased ethanol intake in adolescent rats (compared to adults) is accompanied by decreased ethanol CTA [20]; moreover, CTA magnitude in individual adolescent rats is inversely related to subsequent voluntary ethanol

intake [16]. Together these results suggest an important role for the aversive effects of the drug in suppressing voluntary ethanol consumption. Importantly, these effects are likely to be clinically relevant, as decreased sensitivity to the effects of ethanol, including aversive effects, is predictive of higher levels of binge drinking and higher risk for development of an alcohol use disorder in human populations [21–23].

The neural circuitry through which the aversive effects of ethanol suppress voluntary consumption is not well defined. Evidence supporting a role for the LHb in learning driven by aversive outcomes, including those caused by other drugs of abuse, raises the possibility that this brain region could contribute to these suppressive effects. To begin characterizing the role of the LHb in ethanol-directed behaviors, we studied voluntary ethanol consumption, ethanol self-administration, yohimbine-induced reinstatement of ethanol seeking, and ethanol-induced CTA in LHb and sham-lesioned rats. Our results show that lesions of the LHb increase both voluntary ethanol intake and self-administration, block yohimbine-induced reinstatement of ethanol seeking, and attenuate a taste aversion conditioned by a single noncontingent ethanol injection.

Materials and Methods

Ethics statement

All procedures used were approved by the University of Utah Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Subjects

136 male Long-Evans rats (300–350 g at the time of receipt; Charles-River, Wilmington, MA,) were used in the present experiments. Rats were single-housed in Plexiglas tub cages and maintained on a 12 hour light/dark cycle. *Ad libitum* access to food and water was available throughout all experimental procedures. A summary of the experimental groups and the timeline of experimental procedures (described below) within each group is provided in Table 1.

Drugs

Ethanol (Decon Labs, King of Prussia, PA) solutions were prepared in filtered tap water to a concentration of 20% (v/v) for use in the intermittent ethanol access paradigm, and prepared in physiological saline to a concentration of 20% for the CTA experiment. Saccharin, quinine and yohimbine (Sigma Aldrich, St. Louis, MO) solutions were prepared in distilled water.

Electrolytic lesion of the LHb

Surgical anesthesia was induced and maintained with isoflurane (5% and 2%, respectively). The skull was exposed and burr holes drilled bilaterally above the LHb. Lesions were produced by passing current (0.5 mA, 10 s) through a stainless steel electrode (AM Systems, Sequim, WA) at two sites within each hemisphere that targeted anterior and posterior portions of the LHb. Coordinates for these sites (in mm from bregma) were: –3 and –3.7 posterior; 0.7 lateral; and –5.4 ventral. In sham-lesioned animals, electrodes were lowered to stereotaxic coordinates 1 mm dorsal to the LHb but no current was passed at the target site. Rats were allowed to recover for at least one week after surgery before experiments commenced.

Voluntary ethanol consumption

Voluntary ethanol consumption was measured using an intermittent ethanol access (IEA) two bottle choice paradigm [24–26]. Rats (n = 34; 17 sham and 17 lesioned) were given 24 hour access to 20% (v/v) ethanol and water in a two bottle choice paradigm in the home cage 3 times per week (Monday, Wednesday and Friday). Ethanol bottles were placed in home cages at 9 a.m. on access days. Ethanol and water bottle positions within each cage were alternated in successive drinking sessions to minimize the effect of side preferences. On days in which ethanol was not presented, *ad lib* water was available. IEA was provided for a minimum of 8 weeks (24 drinking sessions). Ethanol and water intake were measured by weighing bottles before and after each 24 hour access period. These measures were used to calculate ethanol intake (normalized to weight, g/kg/24 h) and preference (ethanol intake/total fluid intake). Body weight and food intake were measured weekly during IEA.

In a subset of sham and lesioned rats (n = 7 rats in each group; see Table 1), the timeline of ethanol intake over 24 hour access periods was investigated by measuring intake in the first hour of ethanol access (9–10 am), the remainder of the light cycle (10 am – 6 pm), the first hour of the dark cycle (6–7 pm), and the remainder of the 24 hour period (7 pm – 9 am). These measurements were

Table 1. Summary of experimental groups and timeline of procedures. Numbered experiments indicated the order in which experiments within each group were carried out.

Rat group	Number of rats		Experiments
	Sham	Lesioned	
1	7	7	1) Intermittent ethanol access
			2) 24 h timeline of ethanol intake
			3) Effects of abstinence on IEA intake
			4) Taste preference
2	10	10*	1) Intermittent ethanol access
			2) Operant ethanol self-administration, extinction, and reinstatement
3	7	8	1) Operant sucrose self-administration, extinction, and reinstatement
4	37	42	1) Ethanol conditioned taste aversion
5	4	4	1) Ethanol metabolism

* A single lesioned rat died during intermittent ethanol access; the 9 remaining lesioned rats were trained in operant ethanol self-administration.

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carried out in rats that had IEA for 9 weeks (27 drinking sessions). In the same group of rats, the stability of ethanol intake after a period of abstinence was investigated. After an initial period of 10 weeks of IEA, rats were subjected to nearly 7 weeks (46 days) without ethanol access. IEA was then restored for two additional weeks to allow comparison of ethanol intake before and after abstinence.

Taste preference: two-bottle choice for saccharin and quinine solutions

Preference for saccharin solutions (0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 mM concentrations) and aversion to quinine solutions (0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 mM) were assessed using a two-bottle choice paradigm in the home cage in a subset of sham and lesioned rats (n = 7 rats in each group). In this paradigm, a single bottle of tastant was made available for 48 hours in the home cage, concurrent with a single bottle of water; intake of the tastant and water were measured every 24 hours, and bottle positions were switched after the first 24 period. Intake during the two 24 hour periods was averaged to produce a single measure of consumption for each tastant concentration. Tastants were presented in sequential order of increasing concentration in consecutive 48 hour intervals. Intake during access to the quinine concentration series was measured first, followed by intake during presentation of the saccharin concentration series. To investigate the timeline of saccharin consumption over 24 hour access periods, intake of highly preferred 5 mM saccharin was measured during the four intervals in which ethanol intake was measured: 9–10 am, 10 am–6 pm, 6–7 pm, and 7 pm–9 am.

Operant responding for ethanol

After a minimum of two months home cage access to 20% ethanol in the IEA paradigm, rats (n = 10 sham and 9 lesioned rats) were trained to self-administer 20% ethanol. Operant chambers (Med Associates, St. Albans, VT) were equipped with a central reward receptacle flanked by retractable levers, with illuminated cue lights over each lever. Operant chambers were enclosed in sound attenuating chambers equipped with fans that increased ventilation and provided masking noise. Ethanol was delivered to the reward receptacle via a programmable syringe pump.

In an initial overnight session, rats were trained on an FR1 schedule to respond for 20% ethanol. In this overnight session, only the active lever was presented to facilitate learning. Each lever press caused the active lever to retract, the associated cue light to extinguish, and resulted in immediate delivery of 0.1 mL 20% ethanol into the reward receptacle. After 5 seconds, the active lever was extended and the cue light illuminated. After the initial overnight session, rats were trained in 1 hour sessions three times per week (Monday, Wednesday, Friday) using an FR1 schedule in which only the active lever was available. After 2 weeks, rats progressed to an FR3 schedule in which the session length was decreased to 30 minutes and the inactive lever was introduced. Lever presses on the inactive lever were recorded but had no programmed consequences. Responses in this paradigm were measured over two weeks before extinction training began (below).

Extinction and reinstatement of ethanol seeking

During extinction sessions, the syringe containing 20% ethanol was removed from the syringe pump, and thus active lever presses no longer resulted in ethanol delivery. In all other respects, the extinction paradigm was identical to the final operant response paradigm described above, including presentation of visual and auditory cues (cue light extinguishment, lever retraction, and syringe pump activation). Extinction sessions were run on alternate weekdays for four successive sessions before testing for yohimbine-induced reinstatement.

Reinstatement was studied by administration of the $\alpha 2$ receptor antagonist yohimbine (2 mg/kg, IP) or vehicle solution (distilled water) 30 minutes prior to testing in extinction sessions. Because yohimbine reliably induces multiple reinstatements of ethanol seeking [27], reinstatement in each animal was measured after each of two yohimbine injections to minimize variability in behavioral results. During reinstatement testing, rats were first tested for operant responding in an extinction session after injection of the vehicle solution. This was followed by a rest day (no injection or operant session). The next day, reinstatement of ethanol seeking after yohimbine injection was tested. Two additional extinction sessions followed, and then an identical testing schedule (vehicle injection, rest day, yohimbine injection) was carried out. For each animal, responses were averaged across each of the two drug administrations to yield a single measure of operant responding after vehicle administration and after yohimbine administration.

Sucrose: Operant responding, extinction and reinstatement

Fifteen ethanol-naive rats (7 sham and 8 lesioned rats) were trained to operantly self-administer 2% sucrose. With the exception of the use of sucrose as a reinforcer, the paradigm used was identical to the final operant paradigm used in ethanol self-administration (30 minute FR3 task with both active and inactive levers available). Training in this group was similar to that described for ethanol self-administration above, and began with an initial overnight training session (FR1, no inactive lever). In five subsequent sessions, the response requirement was maintained at FR1 but the session duration was shortened to a single hour. Finally, rats in this group progressed to the final paradigm (FR3, half hour duration, inactive lever introduced) for ten successive sessions.

Thereafter, extinction followed by reinstatement sessions were carried out. The extinction paradigm was identical to that described for extinction of ethanol seeking. A total of seven successive extinction sessions were run in this experiment. This increase (over four sessions used in extinction of ethanol seeking) was incorporated because response rates for sucrose self-administration were substantially higher than those occurring during ethanol self-administration. Yohimbine-induced reinstatement was tested in this experiment in a manner identical to that described above in studies of ethanol reinstatement.

Conditioned taste aversion

A total of 79 ethanol-naïve animals (37 sham and 42 lesioned male Long-Evans rats) were included in the CTA experiment. After lesion surgery, all rats were single housed and allowed at least one week recovery in home cages. Thereafter, rats were habituated to handling for two days before beginning the CTA experiment. Throughout the experiment, rats were maintained with *ad lib* access to both food and water. We avoided water deprivation often used in CTA paradigms because dehydration causes anorexia [28]. Food deprivation has been shown activate LHb neurons [29,30], raising the concern that water deprivation and associated dehydration-induced anorexia might differentially affect motivation in sham and LHb-lesioned rats. To induce consumption, a highly palatable supersaccharin solution (0.125% saccharin + 3% glucose) was used as the conditioned stimulus.

The first day of the CTA experiment, rats were given 30 minutes home cage access to supersaccharin. Rats were then assigned to saline or ethanol injection groups, with mean supersaccharin consumption matched between groups. Rats in the resulting four groups (LHb lesion/sham x saline/ethanol injection) were immediately injected with either ethanol (0.7 g/kg of 20% ethanol, IP) or saline (volume matched to ethanol injections) and returned to their home cages. The ethanol dose used was based on pilot studies showing it produced reliable but not saturating CTA. On each of the next six days, rats again received a single period of 30 minutes home cage access to the supersaccharin solution and consumption was measured. Supersaccharin access sessions, including the initial session, occurred between 4 and 6 pm daily.

Measurement of blood ethanol concentration

Blood ethanol concentration (BEC) was measured after voluntary ethanol intake in the IEA paradigm and operant self-administration sessions. BECs after ethanol consumption in the IEA paradigm were measured from tail vein blood collected after the first 30 minutes of ethanol access, the interval during which previous results [31] and our own measurements suggested intake rates were highest. BECs were measured in rats that had received 14 weeks of IEA. BECs after operant responding for ethanol were measured in tail vein blood collected immediately after 30 minute self-administration sessions.

To determine if LHb lesion altered ethanol metabolism, we measured BECs after noncontingent injection of ethanol (1 g/kg 20% ethanol, IP) in a separate group of ethanol-naïve rats (4 sham and 4 lesioned).

Blood samples were collected into heparinized capillary tubes at 15, 30, 60, 120 and 180 minutes after injection. For all BEC measurements, blood plasma was isolated from samples by perchloric acid precipitation and brief centrifugation (2000 rpm, 5 m). BEC was measured using the NAD-NADH enzyme spectrophotometric method [32,33].

Analysis of lesions

Rats were deeply anesthetized with pentobarbital and perfused with physiological saline followed by 4% formaldehyde. Brains were cryoprotected and sectioned in 50 μm slices. Sections were mounted and Nissl stained. Damage to the LHb was localized by comparison to a reference atlas [34] by an observer blind to behavioral results. Two lesioned rats died during or shortly after experimental procedures and lesion sites were not analyzed in these animals.

Though damage was largely confined to the LHb, some lesion sites encroached upon surrounding structures, including the medial habenula (MHb). To determine if damage to this structure contributed to voluntary ethanol intake in the IEA paradigm, we quantified the extent of this damage in each lesioned animal. The damage to the MHb in each hemisphere was estimated by visual inspection, and was scored as 0% (no damage), 25%, 50%, 75%, or 100% (complete ablation). Scores for each hemisphere were averaged to produce a single estimate of damage to the MHb within each rat. Rats were divided into high and low damage groups by performing a median split of this data, and ethanol intake in the IEA paradigm was then compared between groups.

Statistical analysis

Voluntary ethanol intake, escalation of ethanol intake, taste preference, and reinstatement of ethanol and sucrose seeking were analyzed using two-way repeated measures ANOVA. All analyses included lesion (sham or LHb) as one factor. The second factor consisted of IEA drinking session (voluntary ethanol intake); time interval (escalation of intake); tastant concentration (taste preference); or drug (reinstatement experiments; yohimbine or vehicle treatment). Extinction of ethanol-self administration and ethanol-induced CTA were analyzed using two- (factors of lesion and time) and three-way (factors of lesion, drug and time) ANCOVA, respectively. Baseline ethanol self-administration and supersaccharin intake were used as covariates in these analyses, respectively. Where appropriate, Holm-Sidak posthoc tests were used. BECs and operant self-administration were analyzed using Pearson's correlation test and t-tests.

Results

Voluntary ethanol consumption

Intermittent home cage access to 20% ethanol (IEA) in a two bottle choice paradigm resulted in steady escalation of ethanol consumption in both sham and lesioned animals (Fig. 1A; main effect of drinking session, $F(23, 728) = 28.2$, $p<0.001$). However, intake in the lesioned group diverged from that in the sham group after the first week (drinking sessions 1–3), escalated more rapidly over the course of the next several weeks, and plateaued at higher stable levels of ethanol intake (main effect of lesion, $F(1, 32) = 9.3$, $p<0.01$; significant interaction of lesion x drinking session, $F(23, 728) = 3.6$, $p<0.001$). Differences in ethanol consumption between sham and lesioned animals reached significance during the seventh drinking session and remained significant from the tenth through the final drinking session ($p<0.05$, posthoc tests). Ethanol consumption in the final drinking session averaged 4.1 ± 0.5 and 6.0 ± 0.5 g/kg/24 h for sham and lesioned animals, respectively. Differences in these normalized measures of ethanol intake were due solely to different levels of ethanol consumption, as mean rat weight did not differ between the two groups during IEA (data not shown; no significant effect of lesion on body weight, $F(1, 32) = 0.6$, NS; and no significant interaction of lesion x time, $F(7, 224) = 0.7$, NS; also no significant effect of lesion on food intake $F(1, 8) = 0.0$, NS; and no significant interaction of lesion x time, $F(8, 96) = 0.9$, NS). Similar differences were apparent in measures of ethanol preference (Fig. 1B), which averaged $43\pm5\%$ and $60\pm5\%$ in sham and lesioned animals, respectively, in the final drinking session (main effect of session, $F(23, 728) = 32.2$, $p<0.001$; main effect of lesion, $F(1, 32) = 11.0$, $p<0.01$; significant interaction of lesion x session, $F(23, 728) = 2.9$, $p<0.001$). Posthoc testing indicated that differences between sham and lesioned rats were significant in drinking sessions 5, 7–8, and 10–24 ($p<0.05$, posthoc tests).

Water consumption in both groups decreased progressively over the course of drinking sessions (Fig. 1C; main effect of session, $F(23, 736) = 26.7$, $p<0.001$). Intake in lesioned rats was significantly lower than that in sham rats (main effect of lesion, $F(1, 32) = 5.1$, $p<0.05$; significant interaction of lesion x session, $F(23, 736) = 1.7$, $p<0.05$). Differences in water intake reached significance first in the 14^{th} drinking session and were intermittently significant thereafter ($p<0.05$, posthoc tests). Total fluid intake (water plus ethanol consumption) did not differ between the two groups (Fig. 1D; no significant effect of lesion, $F(1, 32) = 0.62$, NS; no significant interaction of lesion x session, $F(23, 736) = 1.2$, NS).

To determine if differences in voluntary ethanol consumption resulted in different blood ethanol concentrations (BECs), we measured ethanol concentration in tail vein blood collected after the first 30 minutes of ethanol access in the IEA paradigm. BEC measures were significantly correlated with ethanol consumption occurring during this period for both sham and lesioned rats (Fig.

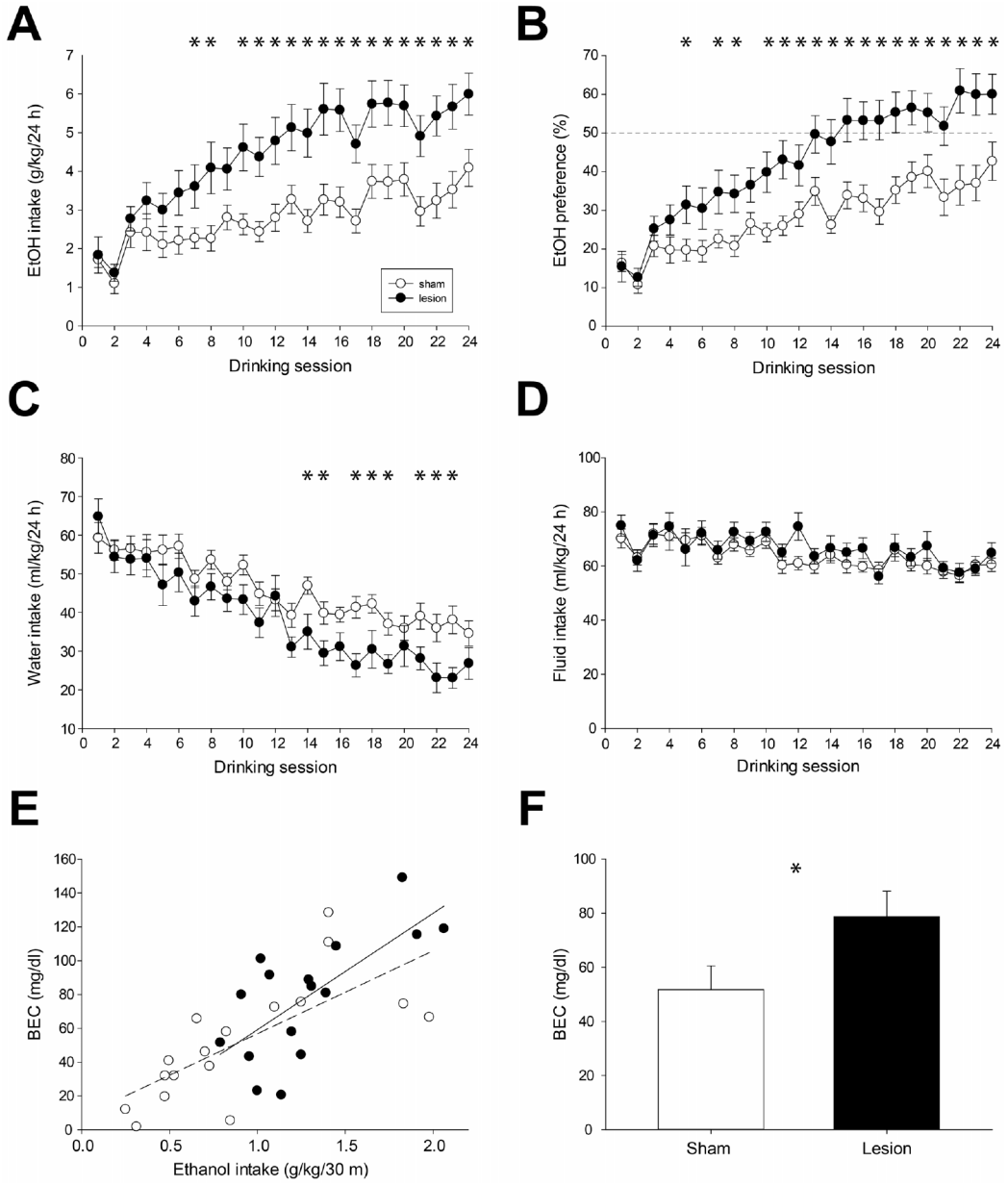

Figure 1. Voluntary ethanol consumption during intermittent access. (A) Lesioned rats consumed significantly more 20% ethanol over the course of 2-bottle choice drinking sessions (3 sessions/week for 8 weeks, 24 sessions total). Symbols indicate mean ethanol intake ± SEM. In this figure and Figures 2–5, filled symbols indicate lesioned animals, open symbols indicate sham animals, and asterisks indicate significant differences ($p<0.05$). (B) Alcohol preference in lesioned rats was significantly higher than that in sham rats. (C) Water intake decreased progressively in both groups, and rats in the lesioned group consumed significantly less water starting in the 5th week (14th session) of the paradigm. (D) Total fluid intake (water + ethanol) did not differ significantly between the two groups. (E) Blood ethanol concentrations (BECs) were significantly correlated with voluntary ethanol intake for both groups. Broken line indicates linear fit for sham group; solid line indicates linear fit for lesioned group. (F) Mean (± SEM) BEC measured after the first 30 minutes of 20% ethanol access was significantly higher in lesioned vs. sham rats ($p<0.05$).
doi:10.1371/journal.pone.0092701.g001

1E; $r^2 = 0.49$ and 0.52 for lesioned and sham rats respectively, $p < 0.01$ for each group). Lesioned rats consumed more ethanol during this period (mean ± SEM of 1.3±0.1 vs. 0.9±0.1 g/kg, $p<0.05$) and reached significantly higher BECs (Fig 1F; 79±9 vs. 52±9 mg/dl, $p<0.05$).

To determine if LHb lesion altered ethanol metabolism, we measured BECs at various time points after a single ethanol injection (1 g/kg 20% ethanol, IP; BEC measured 15, 30, 60, 120, and 180 m after injection) in a separate group of previously ethanol-naïve rats (n = 4 sham and 4 lesioned). BECs in these groups did not differ at any time point after ethanol injection (Table 2; no effect of lesion, $F(1, 6) = 0.04$, NS; no interaction of time x lesion, $F(6, 24) = 0.9$, NS), suggesting that lesion of the LHb had no effect on ethanol metabolism.

Average ethanol consumption in the first week did not differ between the two groups, though the lesioned group drank slightly more (Fig 2A; 2.1±0.3 vs. 1.7±0.2 g/kg/24 h in the lesioned and sham groups respectively, NS). By comparison, ethanol intake in the lesioned group was substantially higher than that in the sham group after eight weeks of IEA (Fig. 2B; 5.9±0.5 vs. 3.8±0.4 g/kg/24 h in the lesioned and sham groups respectively, $p < 0.01$). This higher level of intake was achieved by a more rapid escalation of intake in the lesioned group that persisted for approximately the first 5 weeks of ethanol access before intake levels plateaued thereafter (Fig. 1A). Analysis of the slope of increasing ethanol intake in these two intervals (first 5 weeks [sessions 1–15] vs. the last 3 weeks (sessions 16–24) of IEA) showed that the slope was significantly higher in the lesioned group during the first 5 weeks of IEA, but not thereafter (Fig. 2C; significant interaction of lesion x interval, $F(1, 32) = 7.9$, $p<0.01$; $p<0.05$, posthoc comparing slope of ethanol intake for sham vs. lesioned rats in sessions 1–15). Following the initial period of rapid escalation of ethanol intake, the rate of change in ethanol intake did not differ in lesioned and sham rats (no significant difference between sham and lesioned groups in sessions 16–24, posthoc NS).

To examine the stability of differences in ethanol consumption between the sham and lesioned rats, a subset of rats (n = 7/group) were allowed an initial 10 weeks of IEA, followed by nearly 7 weeks of abstinence (46 days), and then two additional weeks of IEA. Rats were maintained in their home cages during the period of abstinence with *ad lib* food and water supplied as usual. Analysis of 20% ethanol intake showed that lesioned rats consumed more ethanol (Fig. 2D-E; main effect of lesion, $F(1, 12) = 6.5$, $p<0.05$), and that this difference was stably maintained before and after the period of abstinence (sham rats – 4.8±1.0 before and 4.4±0.7 g/kg/24 h after; lesioned rats – 7.2±0.8 before and 6.7±0.7 g/kg/24 h after; no significant effect of time, $F(1, 12) = 3.6$, NS; no significant interaction of lesion x time, $F(1,12) = 0.01$, NS).

Taste preference

Gustatory function contributes to voluntary ethanol intake [35] and habenular lesions have been reported to alter bitter taste aversion [36]. We therefore investigated bitter and sweet taste preference in home-cage two bottle choice experiments in sham and lesioned rats. Quinine preference decreased as a function of concentration in both groups of rats (Fig. 3A; main effect of quinine concentration, $F(6, 12) = 58.7$, $p<0.001$), but did not differ between sham and lesioned rats (no significant effect of lesion, $F(1, 12) = 0.3$, NS; no significant interaction of concentration x lesion, $F(6, 72) = 0.3$, NS).

Saccharin preference increased as a function of increasing concentration (Fig. 3B; main effect of saccharin concentration, $F(6, 12) = 54.6$, $p<0.001$). There was no significant difference between sham and lesioned groups (no significant effect of lesion, $F(1, 12) = 2.4$, NS). Preference for saccharin appeared to be higher at highly preferred saccharin concentrations (0.5 mM and above) but this did not reach significance (no significant interaction of lesion x saccharin concentration, $F(6, 72) = 1.5$, NS). Ceiling effects may have obscured differences, however, as preference in both groups was near maximal at concentrations of 0.5 mM saccharin and above. Inspection of the volume of saccharin consumed showed that saccharin intake was significantly higher for lesioned rats at preferred concentrations (Fig. 3C; significant interaction of saccharin concentration x lesion, $F(6, 72) = 5.9$, $p<0.001$). Lesioned rats consumed significantly more than sham rats at saccharin concentrations of 0.5 mM and above ($p<0.05$, posthoc tests).

To further investigate elevated saccharin and ethanol intake in lesioned animals, we studied the timing of 5 mM saccharin and 20% ethanol ingestion during 24 hour periods of home cage access. The distribution of saccharin intake across the 24 hour cycle was similar for sham and lesioned rats, with both groups consuming saccharin at highest rates during the first hour of the dark cycle (Fig. 3D, left panel; main effect of time, $F(1, 12) = 57.0$, $p<0.001$; drinking rate during 1st hour of dark cycle [6–7 pm] significantly higher than that occurring during all other intervals, $p<0.05$, posthoc). Though lesioned rats consumed more saccharin than sham rats in every time period except the first hour of access, this difference did not reach statistical significance (no main effect of lesion, $F(1, 12) = 3.2$, NS; and no significant interaction of lesion x time, $F(3, 36) = 1.2$, NS). By contrast, lesioned rats consumed significantly more ethanol specifically during the first hour of ethanol access (Fig. 3D, right panel; significant interaction of lesion x time, $F(3, 36) = 2.9$, $p<0.05$; lesioned vs. sham intake in 1st hour of access, $p<0.05$, posthoc). Interestingly, these and previous results [31] suggest that the highest rates of voluntary ethanol intake leading to peak BECs occur within the first hour of access, and suggest that elevated ethanol intake in lesioned rats may be motivated by the pharmacological effects of the drug. Notably, rates of saccharin intake during the first hour of access were nearly identical in sham and lesioned animals.

Operant self-administration of 20% ethanol

A total of 19 rats (10 sham and 9 lesioned rats) with a history of IEA were trained to self-administer 20% ethanol on an FR3 schedule. Stable levels of responding (average of last three

Table 2. BECs (mg/dl) in ethanol naïve sham and lesioned rats following 1 g/kg IP ethanol injection.

	Time (minutes) after ethanol injection				
	15	**30**	**60**	**120**	**180**
Sham	129±15	118±7	108±9	76±11	31±11
Lesioned	118±18	108±14	109±11	77±4	38±9

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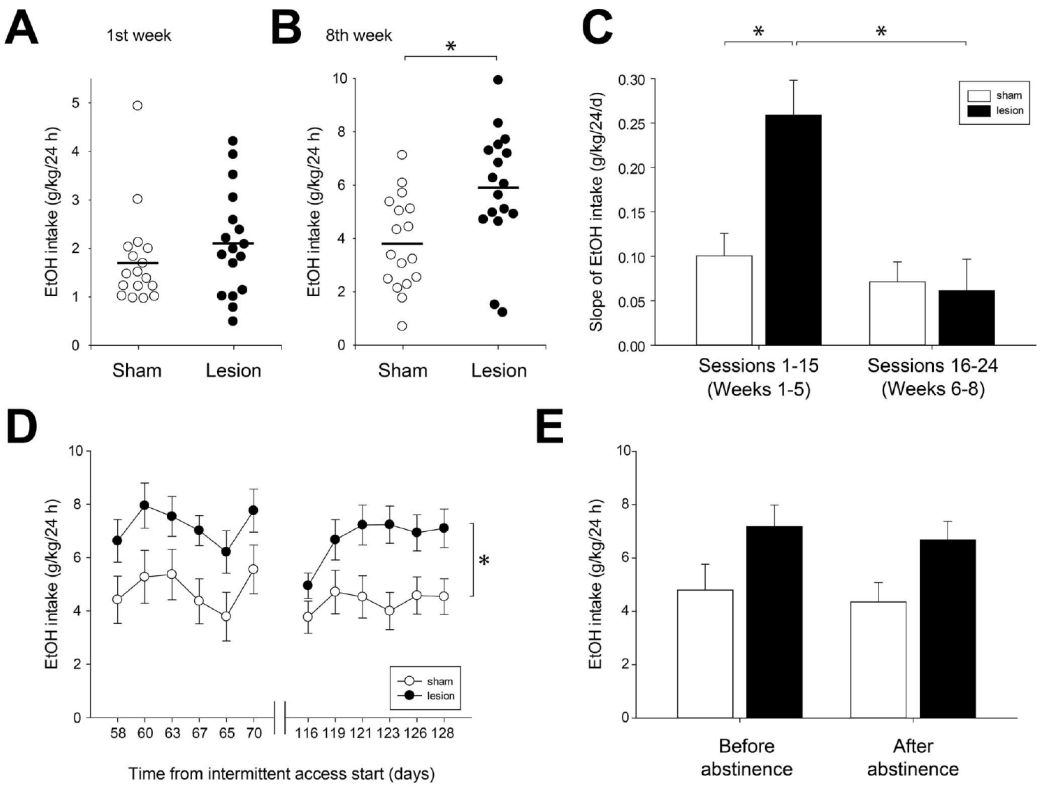

Figure 2. Escalation and stability of voluntary ethanol consumption. (A) Mean ethanol consumption did not differ between sham and lesioned groups in the first week (3 sessions) of IEA. Each symbol indicates the average ethanol intake for a single rat in the first week of IEA. Mean values of each group are indicated by horizontal bars. (B) Lesioned rats drank significantly more ethanol in the eighth week of IEA. (C) Lesioned rats escalated ethanol intake at higher rates than sham rats over the first 5 weeks of IEA (sessions 1–15), but not during the last 3 weeks of access (sessions 16–24). Bars graphs indicate the average slope of ethanol intake over the intervals shown. (D –E). Significant differences in ethanol consumption between groups were stably maintained after a period of abstinence. Rats were withdrawn from IEA for approximately 7 weeks (46 days), and then restored to IEA for an additional two weeks. Lesioned rats drank more than sham rats both before and after this period of abstinence. Asterisk (D) indicates significant main effect of lesion ($p<0.05$) on ethanol intake.
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rewarded sessions) were significantly higher in lesioned vs. sham rats (Fig. 4A; 87±15 vs. 50±10 lever presses for lesioned and sham rats, respectively; $t = 2.1$, $p<0.05$). In addition, levels of ethanol intake were higher in lesioned animals (Fig. 4B; 0.68±0.12 vs. 0.37±0.07 g/kg for lesioned and sham rats; $t = 2.3$, $p<0.05$). Increased lever pressing in lesioned rats was sustained for the duration of each operant session (Fig. 4C; main effect of lesion, $F(1, 17) = 5.9$, $p<0.05$; main effect of time, $F(14, 238) = 20.5$, $p< 0.001$; no significant interaction of lesion x time, $F(14, 238) = 0.6$, NS). BECs measured immediately after 30 minute operant response sessions revealed that lesioned rats achieved higher BECs during self-administration sessions (Fig. 4D; 42±12 vs. 13±4 mg/dl for lesioned and sham rats, $p<0.05$).

Operant self-administration of 2% sucrose

To determine if increased operant responding in lesioned rats occurred selectively for 20% ethanol, we studied self-administration of 2% sucrose. Sham and lesioned rats reached similar levels of stable operant responding for sucrose (Fig. 4E; 338±84 vs. 363±64 responses per session for sham and lesioned rats, respectively; $t = 0.2$, NS). In addition, the pattern of operant responding occurring within sessions did not differ between groups (Fig. 4F; significant main effect of time, $F(14, 182) = 29.4$, $p< 0.001$; but no significant effect of lesion, $F(1, 13) = 0.4$, NS; and no significant interaction of lesion x time, $F(14, 182) = 0.7$, NS).

Extinction and reinstatement of ethanol seeking

After rats reached stable levels of operant responding for ethanol, self-administration was extinguished over four successive extinction sessions. Lever pressing in the lesioned group was higher than that in sham rats in the first extinction session (Fig. 5A), but there was no significant interaction of lesion x extinction session ($F(3, 128) = 0.7$, NS) when ethanol self-administration rates were included as a covariate in the analysis (Fig. 5A, "Last rewarded session"). Mean response rates (± SEM) in the last extinction

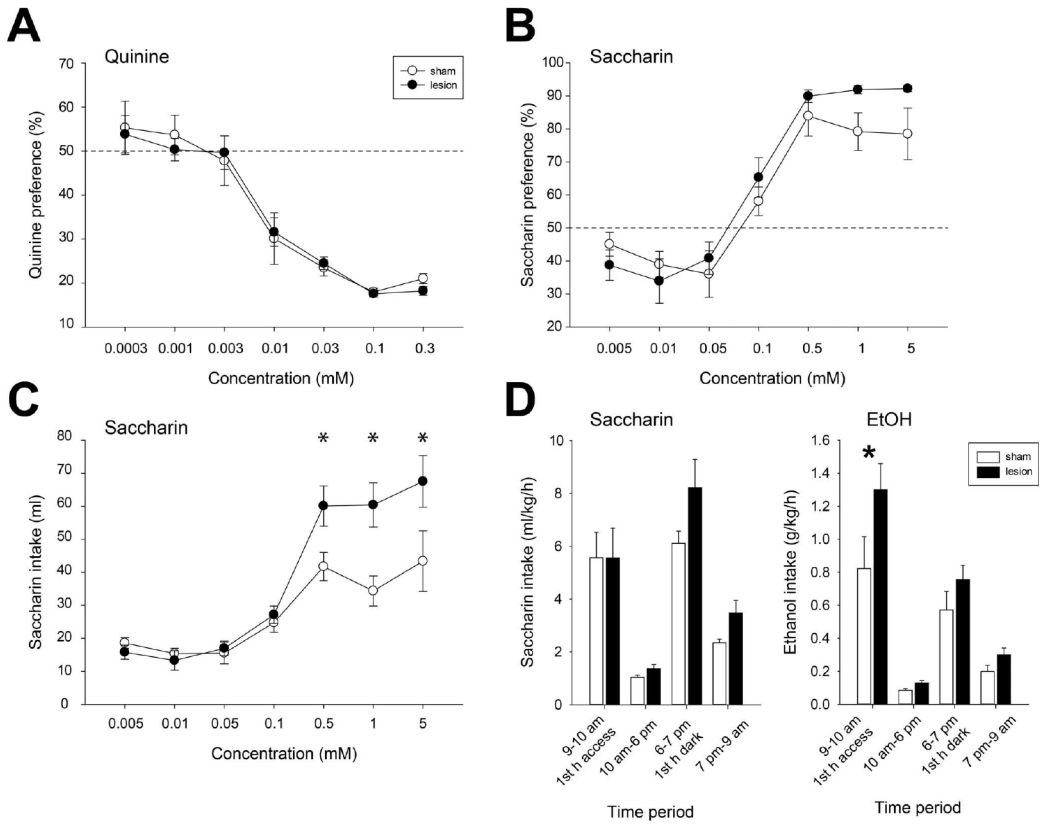

Figure 3. Taste preference. (A) Aversion to quinine did not differ between sham and lesioned rats. (B) Preference for saccharin did not significantly differ between sham and lesioned rats, despite quantitatively increased preference in lesioned rats at saccharin concentrations of 0.5 mM and above. (C) Saccharin intake was significantly higher in lesioned rats at concentrations of 0.5 mM saccharin and above. (D) Timeline of saccharin (left panel) and ethanol (right) intake over 24 hour sessions. The pattern of saccharin intake over 24 hours did not differ between sham and lesioned animals. Rates of intake were similar in the first hour of saccharin access and highest for both groups in the first hour of the dark cycle. By contrast, rates of 20% ethanol intake were higher in lesioned rats specifically during the first hour of access.
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session were 11±3 lever presses for lesioned rats and 14±4 for sham rats.

Reinstatement of ethanol seeking was tested by administration of yohimbine (2 mg/kg) or vehicle (distilled water) 30 minutes before testing in the extinction paradigm. Vehicle administration resulted in rates of operant responding very similar to those recorded in the final extinction session (Fig. 5B; 10±2 vs. 15±3 lever presses in lesioned and sham rats, respectively). Yohimbine caused a robust increase in operant responding in sham rats, but had no significant effect on responding in lesioned animals (18±8 vs. 47±9 lever presses in lesioned and sham rats, respectively; significant interaction of lesion x drug $F(1, 17) = 4.8$, $p<0.05$). Post hoc analyses showed that yohimbine administration increased lever pressing in sham rats relative to the lesioned group and relative to vehicle administration in the sham group ($p<0.05$, posthoc tests). Thus, lesion of the LHb blocked the ability of yohimbine to reinstate ethanol seeking.

Responses on the inactive lever did not differ between groups, nor were they affected by drug administration (Table 3; no effect of lesion, $F(1, 17) = 1.2$, NS; no effect of drug, $F(1, 17) = 0.8$, NS; no interaction of lesion x drug, $F(1, 17) = 3.6$, NS).

Extinction and reinstatement of 2% sucrose seeking

To determine if reinstatement deficits in lesioned animals were specific for ethanol seeking, we studied yohimbine-induced reinstatement of sucrose seeking in ethanol-naïve rats. After animals reached stable levels of 2% sucrose self-administration, responding was extinguished over seven extinction sessions. Lever press responses declined rapidly across extinction sessions (Fig. 5C; main effect of extinction day, $F(6, 78) = 34.7$, $p<0.001$) in both sham and lesioned rats (no effect of lesion, $F(1, 13) = 0.4$, NS; no interaction of lesion x day, $F(6, 78) = 1.0$, NS). Mean response rates in the final extinction session were 16±5 lever presses for lesioned rats and 4±1 for sham rats.

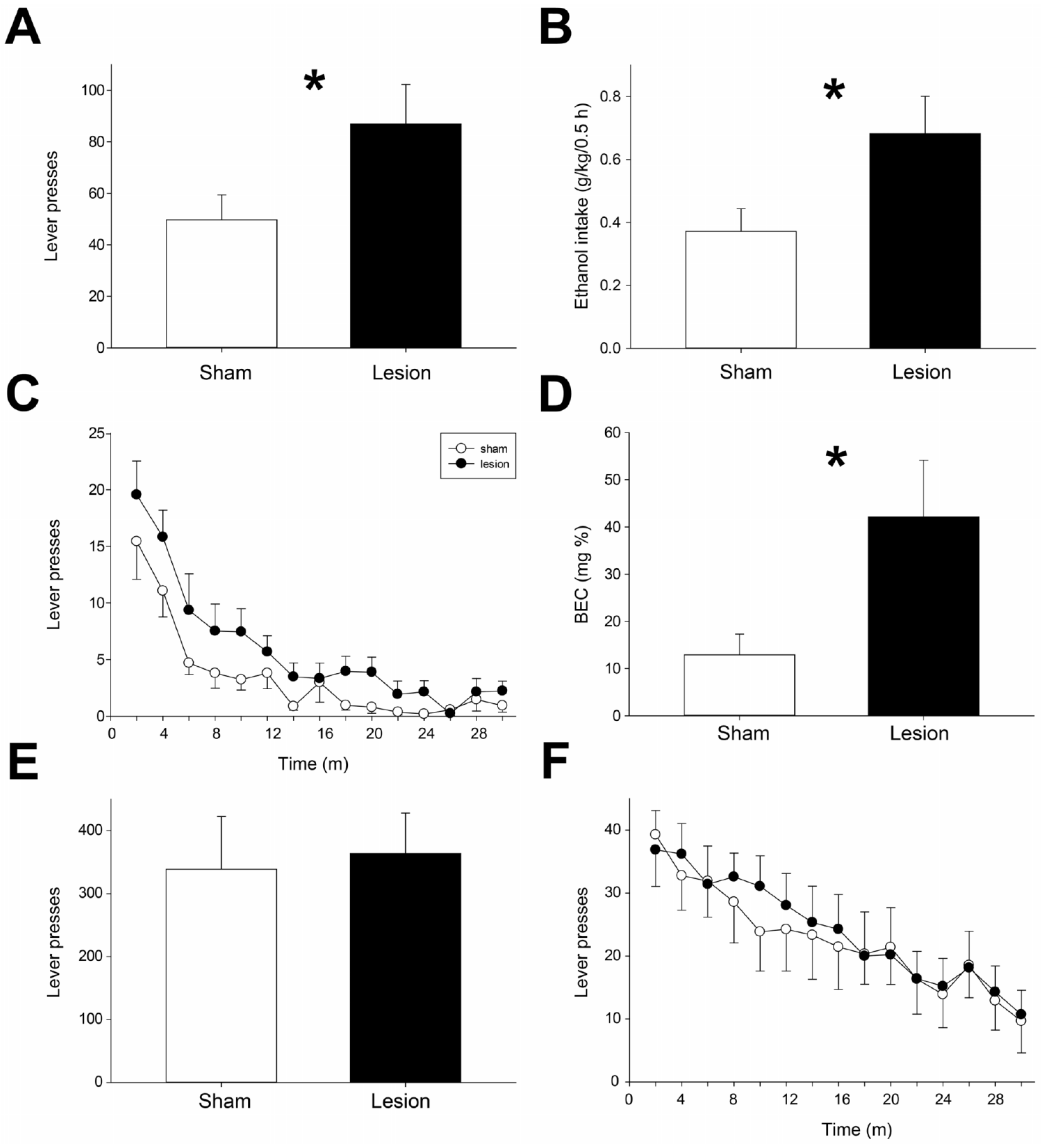

Figure 4. Operant self-administration of ethanol and sucrose. (A-B) Operant responding (A) for 20% ethanol was significantly higher in lesioned vs. sham rats, resulting in elevated levels of ethanol consumption (B). (C) Timing of lever pressing across 30 m self-administration sessions. Compared to sham animals, lesioned rats maintained elevated response rates for the duration of the session. (D) Operant self-administration resulted in significantly higher BECs in lesioned rats compared to sham rats. (E-F) Sham and lesioned rats showed similar levels of operant responding for 2% sucrose (E), and similar patterns of responding over the course of the 30 m session (F).
doi:10.1371/journal.pone.0092701.g004

Similar to the results seen for reinstatement of ethanol seeking, yohimbine reinstated sucrose seeking in sham but not lesioned rats (Fig. 5D; significant interaction of lesion x drug, $F(1, 13) = 6.5$, $p < 0.05$; also main effect of drug, $F(1, 13) = 6.4$, $p < 0.05$). Yohimbine administration resulted in a significant increase in operant responding in the sham group relative to operant responding after vehicle administration (19 ± 4 vs. 5 ± 2 lever presses after yohimbine and vehicle, respectively; $p < 0.05$, posthoc). A trend

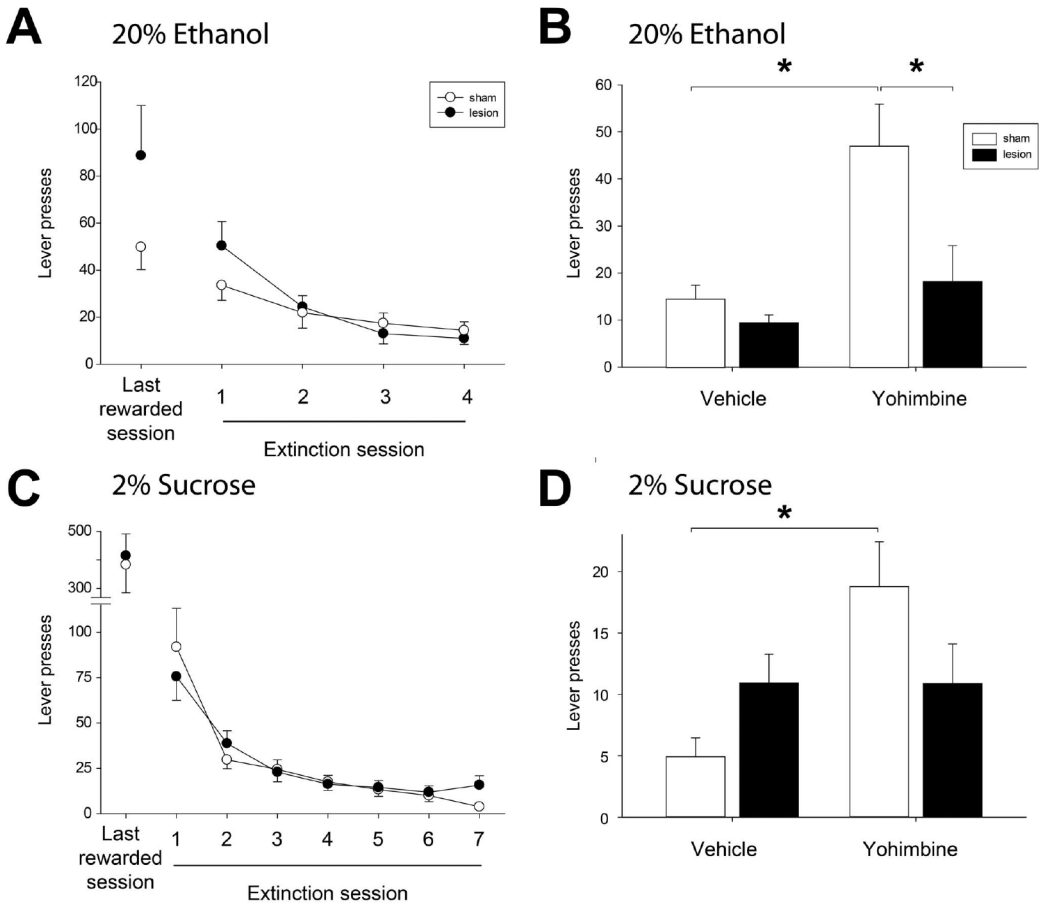

Figure 5. Extinction and reinstatement of ethanol and sucrose seeking. (A) The number of lever presses in lesioned and sham rats did not differ during extinction of operant responding for 20% ethanol. (B) Yohimbine administration reinstated ethanol seeking in sham but not lesioned rats. The number of lever presses by sham rats after yohimbine injection was significantly higher than that occurring after vehicle injection, and higher than lever presses performed by lesioned rats after yohimbine administration. Brackets indicate significant posthoc differences. (C) Sham and lesioned rats showed similar rates of extinction of responding for 2% sucrose. (D) Yohimbine administration induced reinstatement of sucrose seeking in sham but not lesioned rats.
doi:10.1371/journal.pone.0092701.g005

Table 3. Inactive lever presses following vehicle or yohimbine administration during reinstatement of ethanol or sucrose seeking.

	Group	Vehicle	Yohimbine
20% Ethanol	Sham	1.2±0.5	0.8±0.2
	Lesioned	0.9±0.4	2.1±0.7
2% Sucrose	Sham	1.1±0.4	1.3±0.4
	Lesioned	1.7±0.6	1.8±1.2

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toward increased operant responding in sham vs. lesioned rats was apparent after yohimbine administration ($p = 0.06$, posthoc test). Yohimbine did not reinstate sucrose seeking in the lesioned group (11±3 vs. 11±2 lever presses after yohimbine and vehicle, respectively; NS). Inactive lever presses did not differ between sham and lesioned rats, nor were they affected by drug administration (Table 3; no effect of lesion, $F(1, 13) = 0.2$, NS; no effect of drug, $F(1, 13) = 0.0$, NS; no interaction of lesion x drug, $F(1, 13) = 0.2$, NS).

Conditioned taste aversion

To determine if LHb lesion affected learning caused by ethanol's aversive effects, we examined ethanol-induced CTA in lesioned and sham rats. Rats in each group were injected with saline or ethanol (0.7 g/kg, IP) after an initial 30 minute period of supersaccharin access in the home cage. Ethanol administration conditioned an aversion to supersaccharin in both sham and lesioned groups as demonstrated by reduced supersaccharin intake after ethanol injection (Fig. 6; main effect of drug, $F(1, 73) = 40.9$, $p < 0.001$; also main effect of time, $F(3.2, 238.0) = 7.2$, $p<0.001$). However, the magnitude of this aversion was dependent on surgical treatment (significant interaction of drug x lesion, $F(1, 73) = 6.6$, $p<0.05$). Posthoc testing revealed that ethanol-induced CTA was attenuated in lesioned rats relative to sham animals, as supersaccharin consumption was significantly higher in lesioned vs. sham rats after ethanol injection (10.7±0.9 vs 7.7±1.0 ml, respectively; $p<0.05$). Lesioned and sham rats consumed similar amounts of supersaccharin on the first day after ethanol injection (Fig. 6, day 1), but lesioned rats appeared to recover from this initial aversion more rapidly than sham operated rats (consumption on days 2–6). However, there was no significant time-dependent difference in drug effects on sham vs. lesioned groups (no significant drug x lesion x time interaction; $F(3.2, 238.0) = 1.0$, NS). In lesioned and sham rats that received control saline injections, lesioned animals consistently consumed less supersaccharin than sham animals (days 1–6), but this did not reach statistical significance.

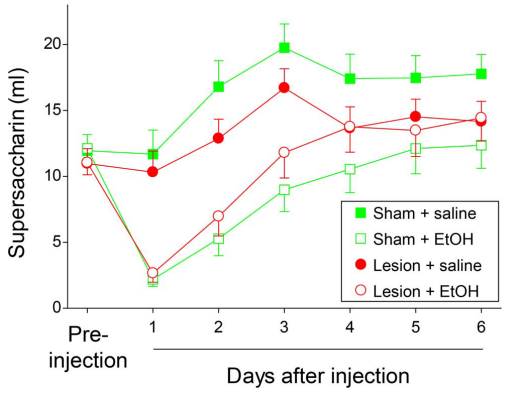

Figure 6. Ethanol induced conditioned taste aversion. LHb lesion attenuated the magnitude of a taste aversion conditioned by a single injection of ethanol. X-axis shows intake of supersaccharin; y-axis shows time of saccharin intake relative to injection in days. Squares and circles indicate sham- and LHb-lesioned rats, respectively. Filled and open symbols indicate saline and ethanol injection groups, respectively.
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Histological confirmation of lesions

Lesions were largely confined to the LHb (Figure 7), though they encroached upon neighboring structures, including the medial habenula (MHb). To determine if lesions of the MHb contributed to increased ethanol intake in lesioned animals, we quantified damage to this structure in lesioned rats tested in the IEA paradigm. Damage to the MHb ranged from a maximum of 50% (complete lesion of the MHb in one hemisphere) to 0%, and averaged 20±5%. Voluntary ethanol intake during IEA did not differ in rats with low vs. high MHb damage (5±2% vs. 33±5% damage, respectively, and ethanol intake of 6.3±0.4 and 5.8±0.9 g/kg/24 h in the last week of IEA in low and high damage groups, respectively; no effect of MHb damage, $F(1, 13) = 0.5$, NS; and no interaction of MHb damage x drinking session, $F(23, 292) = 0.7$, NS), suggesting that damage to this structure did not contribute to the results reported here.

Discussion

Our results show that the LHb plays an important role in controlling ethanol-directed behaviors. LHb lesion increased the rate at which rats escalated voluntary ethanol consumption in a two bottle choice paradigm, resulted in higher levels of maintained intake relative to sham rats both before and after a period of abstinence, and gave rise to higher BECs relative to sham animals. Operant self-administration of ethanol was also increased in lesioned rats, again resulting in higher BECs than those measured in sham animals. In addition, yohimbine-induced reinstatement of ethanol and sucrose seeking were blocked in animals with LHb lesions. Finally, our results show that ethanol CTA was significantly attenuated by LHb lesion. These results demonstrate an important role for the LHb in regulating ethanol-seeking and consumption and moreover show that LHb circuits contribute to learning driven by ethanol's aversive effects.

Effects of LHb lesion on ethanol intake and self-administration

LHb lesions did not acutely increase voluntary ethanol consumption, but rather increased the rate at which escalation of intake occurred, leading to higher sustained levels of ethanol consumption. Ethanol intake in the first week of the IEA paradigm was similar in sham and lesioned groups (Figs. 1A and 2A). Drinking levels in the two groups diverged gradually, with LHb-lesioned rats escalating their intake more rapidly than those in the sham group (Figs. 1A and 2C). Drinking in lesioned rats plateaued around the 6th week of IEA, at which time ethanol intake in lesioned rats was approximately 50% higher than that in the sham group. This difference in intake was maintained for the remainder of the IEA paradigm.

Changes in taste preference are unlikely to contribute to increased ethanol intake and self-administration in LHb-lesioned rats. Though lesions of the habenular complex have been reported to decrease quinine intake [36,37], we found that sham and lesioned rats showed nearly identical dose-dependent aversion to quinine (Fig. 3A). LHb lesion clearly increased consumption of preferred saccharin concentrations (Fig. 3C; concentrations of 0.5 mM and higher). However, patterns of elevated saccharin and ethanol intake differed, suggesting distinct physiological mechanisms underlying consumption of the two solutions (Fig. 3D). Saccharin consumption occurred at highest rates during the first hour of the dark cycle (6-7 pm). In contrast, rates of ethanol intake were highest for both groups during the first hour of access (9-10 am), and were significantly higher in the lesioned group exclusively during this period. Because drinking during this interval is likely to

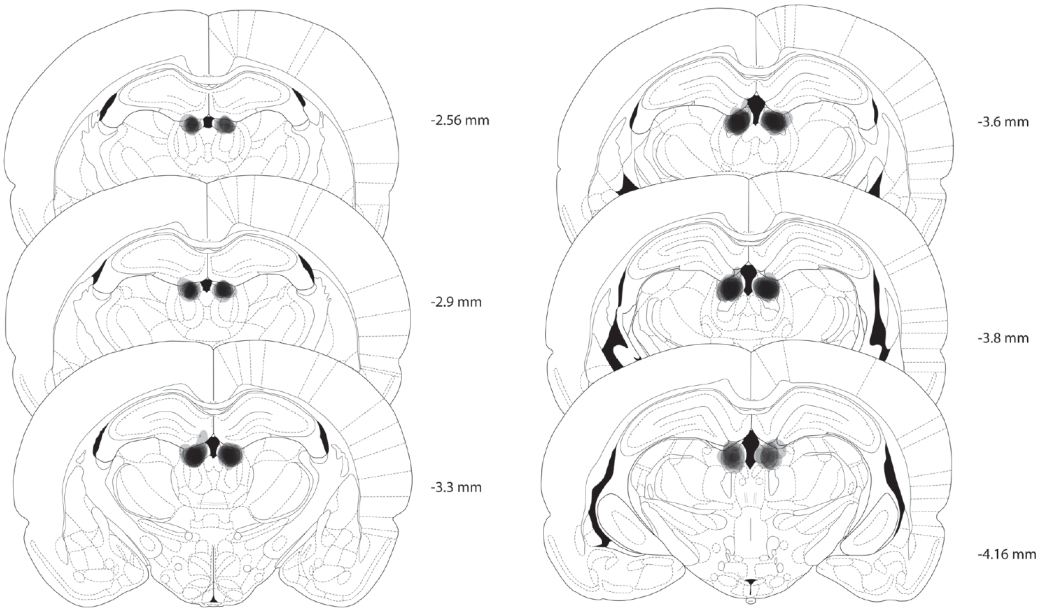

Figure 7. Lateral habenula lesions. Lesion sites were centered on the lateral habenula. Semi-transparent shading shows lesion sites overlaid for each rat, so that darkest areas indicate areas of greatest damage. In some rats, lesions extended to nearby structures (particularly including the medial habenula, medial thalamic areas, and the dentate gyrus). Numbers to the right of each section indicate anterioposterior position relative to bregma.
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result in the highest BECs [31], this result suggests that increased ethanol intake in lesioned rats during this interval may be motivated by the pharmacological effects of the drug.

Our findings of increased ethanol intake and self-administration in LHb-lesioned animals contribute to a growing body of evidence implicating the habenula in regulating drug seeking behaviors. Habenular circuits have been shown to play a central role in regulating learning driven by negative outcomes [6–11], and recent studies have shown that this function importantly extends to regulation of drug-seeking behaviors [6,14,15]. Electrical stimulation of the LHb accelerates extinction of cocaine self-administration and attenuates subsequent cocaine-induced reinstatement [15], consistent with a mechanism in which excitation of LHb neurons suppresses drug-seeking through aversive conditioning. Jhou and colleagues [6] have shown that cocaine induces delayed excitation in a subset of LHb and downstream RMTg neurons, and that optogenetic inhibition of RMTg firing specifically during this period of delayed excitation blocks cocaine-conditioned avoidance behaviors. In addition, studies of mechanisms regulating nicotine intake suggest an analogous role for circuits originating in the medial habenula in mediating aversion-dependent suppression of nicotine self-administration [14].

Increased ethanol intake and self-administration in LHb-lesioned animals may also arise through deficits in learning from aversive drug outcomes. Ethanol causes dose-dependent aversive effects that include nausea, sedation, motoric impairment and hangover effects [16]. These effects are thought to condition a learned aversion to ethanol that acts to decrease subsequent intake, as suggested by the inverse correlation of ethanol CTA and voluntary ethanol intake [18,19]. Our results show that lesion of the LHb causes attenuation of a taste aversion conditioned by a single noncontingent ethanol injection. LHb neurons are known to be excited by negative stimuli, including drug-induced negative stimuli [1,2,6]. Loss of an ethanol-induced aversive signal after LHb ablation could thus contribute to increased ethanol intake in lesioned rats. In this regard, our finding that LHb lesion did not acutely increase voluntary ethanol consumption, but rather increased the rate at which escalation of intake occurred, is suggestive of an impairment in ethanol-induced aversion learning. However, additional experiments are needed to determine if attenuated CTA in LHb-lesioned animals plays a causal role in increasing voluntary ethanol intake.

Effects of LHb lesion on extinction and reinstatement of ethanol and sucrose seeking

Given evidence of an important role for the LHb in aversive conditioning, lesion of this structure might be expected to impair extinction learning, particularly as lesion and stimulation of the LHb respectively impair and accelerate extinction of cocaine-seeking [15,38]. However, we found no extinction deficits in lesioned rats. Our results agree with those reported in a recent study of cocaine self-administration, in which operant responding in an initial extinction test was unaltered by acute pharmacological inactivation of the LHb [39]. These findings contrast with those of a recent study showing that LHb lesions block extinction learning in rats trained to self-administer cocaine [15]. Methodological differences (bilateral electrolytic lesions vs. unilateral excitotoxic lesion) and/or the drug tested could contribute to these divergent results. It is also possible that recovery of neural function may have contributed to intact extinction learning in our lesioned animals, as

operant responding in extinction was tested weeks after lesions were made.

The effects of LHb lesion were not specific to reinstatement of ethanol seeking, as yohimbine-induced reinstatement of sucrose seeking was also blocked in lesioned animals. Our results extend recent findings that pharmacological inactivation of the LHb blocks yohimbine-induced potentiation of cue-dependent reinstatement of cocaine-seeking, as well as attenuating yohimbine-induced anxiety-related behaviors [39]. Habenular neurons, particularly those in the medial subdivision of the LHb, are activated by an array of stressful stimuli, including foot shock, novel environments, restraint stress, food restriction and lithium chloride injection [29,30,40,41]. Habenula lesions impair stress-induced potentiation of prepulse inhibition, decrease avoidance learning tested under high stress conditions, and block the development of learned helplessness in response to inescapable shock [42–44]. Taken together, these results demonstrate an extensive role for habenular function in mediating stress-induced behavioral responses. These results may have clinical implications, as they raise the possibility that individual differences in LHb function could importantly contribute to vulnerability to stress-induced relapse of drug seeking in abstinent alcoholics.

In summary, our study provides novel evidence of an important role for the LHb in a number of ethanol directed behaviors. Our results show that LHb lesions increase voluntary ethanol intake and operant-self administration and block yohimbine-induced reinstatement of ethanol seeking. Further, LHb lesions attenuate ethanol CTA. These results are likely to have important implications for mechanisms underlying alcohol use disorders as studies in human volunteers show that vulnerability to developing an alcohol use disorder is inversely related to sensitivity to acute ethanol effects, including the aversive effects of the drug [21–23].

Author Contributions

Conceived and designed the experiments: SAT. Performed the experiments: AKH CS ALS MSS ST SAT. Analyzed the data: AKH CS SAT. Contributed reagents/materials/analysis tools: AKH CS SAT. Wrote the paper: SAT.

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CHAPTER 5

DISCUSSION

Ethanol-seeking is regulated by mechanisms of reward and aversion

It has long been known that learning driven by positive and negative outcomes contributes to ethanol-seeking behavior, but unanswered questions remain regarding the neural mechanisms of these learning processes. Given the prevalence and severity of alcohol use disorders (AUDs), answering these questions is critically important to advancing our understanding of these disorders. In this dissertation, I described the development of techniques necessary to observe signaling in the brain's reward systems during ethanol-seeking behavior (Chapter 2). Next, I described an investigation that used these techniques to characterize signaling in the brain's reward pathways during different phases of self-administration of ethanol (Chapter 3). Finally, I described experiments identifying a region of the brain that appears to mediate learning about the aversive properties of ethanol and thereby affects intake (Chapter 4). In the following sections I will describe the implications of these findings and discuss possible future directions.

Discussion of phasic DA in motivating ethanol-seeking behavior

Dopamine (DA) plays a critical role in motivating behavior for rewarding stimuli, and therefore is a crucial element in the brains reward circuitry. For example, rapid, phasic release of DA in the nucleus accumbens (NAcc) core occurs during cocaine- and food-seeking behavior (Phillips et al., 2003b; Roitman et al., 2004). Furthermore, stimulation of phasic DA release potentiates cocaine- and food-seeking behavior (Waldbillig, 1975; Phillips et al., 2003b; Steinberg et al., 2013). These results suggest that phasic DA motivates reward-seeking behavior. Despite these findings, it was unknown if phasic DA signaling motivates ethanol self-administration. Previous investigations using FSCV used only noncontingent infusion of ethanol (Cheer et al., 2007; Robinson et al., 2009) or recorded from other brain regions In this dissertation, I described the development of the methodologies necessary for conducting FSCV experiments in self-administering rodents (Chapter 2). These methods were essential to enable recordings of phasic DA during operant ethanol self-administration.

Ethanol predictive cues

In these experiments, I found that phasic DA release was evoked upon presentation of cues predictive of ethanol availability. The presence of DA just after the onset of a predictive cue is consistent with previous findings showing phasic DA responses to cues that predict rewarding stimuli (Ljungberg et al., 1992; Schultz et al., 1997; Day et al., 2007; Wassum et al., 2012a). Furthermore, this result supports findings from the microdialysis literature showing robust increases

in DA during a waiting period prior to ethanol self-administration (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002). Herein, I extend these findings by demonstrating that DA release evoked by anticipatory cues occurs on a rapid time scale, accompanying cue presentation on each behavioral trial. Furthermore, I found that a larger cue-evoked DA transient was predictive of a shorter latency to complete an ethanol-rewarded lever press. These results agree with data showing elevated DA at the beginning of a sequential food-seeking task is predictive of faster responding, as well as data showing that infusions of DA receptor antagonists into the NAcc increase latencies to lever press for food-reward (Nicola, 2010; Wassum et al., 2012a). Furthermore, my results are consistent with theories that DA has a role in mediating the incentive motivation, or "wanting," for reward predictive stimuli. These results suggest ethanol-seeking uses similar mechanisms as other rewarding stimuli (Berridge and Robinson, 1998).

Despite the robust DA release evoked by ethanol-predictive cues in *ad lib* fed rats (Figure 3.3), it should be noted that DA release evoked by predictive cues in food-deprived rats was much smaller and did not reach statistical significance (see Figure 2.4). However, in addition to food deprivation, differences in cue presentation between the two behavioral paradigms might account for this difference in outcome. Specifically, in the food-deprived animals reported in Chapter 2, there were two temporally separated predictive cues. Given the trend towards increased DA signal for both of these cues, it seems possible that different rats may have associated one, but not both, of these cues with ethanol. Indeed,

phasic DA has been observed to be split between multiple predictive stimuli during intermediate stages of training (Wassum et al., 2012a). In later experiments using *ad lib* fed rats, these predictive cues were altered so that they occurred simultaneously rather than sequentially. This change may have contributed to increased DA release in response to ethanol predictive cues.

Lever-press for ethanol reward

I also recorded DA release evoked during the performance of an ethanol-rewarded lever press. This signal was consistent across food-deprived rats studied in Chapter 2 and *ad lib* fed rats studied in Chapter 3. In both of these cases, the DA began to increase about 1 second before the lever press and peaked several hundred milliseconds after the lever press. Similar signals have been observed in rats lever pressing for cocaine and food, suggesting that the role of DA signaling during ethanol-seeking is similar for many other types of rewarding stimuli (Phillips et al., 2003b; Roitman et al., 2004). Further, the performance of goal-approach behaviors has been proposed to be dependent on DA signaling (Ikemoto and Panksepp, 1999), with subsequent work hypothesizing that such DA signaling mediates the selection of the unique set of motor programs required for the approach behavior (Nicola, 2007). My results are consistent with both hypothesis, and suggest signaling dynamics occur that are also consistent with these hypotheses during ethanol-seeking.

Ethanol consumption

Lastly, I found that ethanol consumption does not appear to evoke phasic DA release in *ad lib* fed rats. It is possible that the extensive training in the operant task may have attenuated DA release evoked by ethanol consumption. Indeed, extensive training has been shown to attenuate DA signaling evoked by primary reinforcers in both classical conditioning and instrumental paradigms (Ljungberg et al., 1992; Schultz et al., 1997; Day et al., 2007). However, my results are at odds with many studies using *in vivo* microdialysis that show robust DA release being evoked during periods of ethanol consumption (Weiss et al., 1993; Gonzales and Weiss, 1998; Melendez et al., 2002; Doyon et al., 2003). This difference may arise because *in vivo* microdialysis is thought to only record slowly changing, tonic DA levels in the extracellular fluid (Watson et al., 2006; Schultz, 2007). This difference leaves open the intriguing possibility that ethanol consumption may be exclusively stimulating release of tonic DA, which is undetectable with fast scan cyclic voltammetry (FSCV). Indeed, studies using noncontingent infusion of ethanol while recording with FSCV and microdialysis found that tonic DA is potentiated more consistently than phasic DA (Robinson et al., 2009). These results suggest that consumption evoked DA release reported in the microdialysis literature may be tonic in nature. As tonic and phasic DA have been proposed to activate different DA receptor subtypes (Gonon, 1988; Dreyer et al., 2010), they also may differentially influence the strengths of inputs to the NAcc. In this way, tonic DA has been proposed to reduce the effect of prefrontal cortex (PFC) input on the NAcc while phasic DA increases the effect of input from limbic regions (Goto and

Grace, 2005). Authors have linked this reduction of PFC and potentiation of limbic input to the perseverative drug taking that is seen in many substance abuse disorders, including AUD (Grace et al., 2007).

While no increase in phasic DA signaling was recorded in rats with *ad lib* access to food, significant phasic DA signaling was observed in association with ethanol consumption by food-deprived animals. Palatable food consumption is well-known to evoke phasic DA signaling (Ljungberg et al., 1992; Schultz et al., 1997; Day et al., 2007; Brown et al., 2011). Further, ghrelin, a neuropeptide associated with hunger, has been found to potentiate phasic DA release evoked by sucrose consumption (Cone et al., 2014). This evidence suggests the possibility that ethanol-evoked phasic DA release in food-deprived animals may be potentiated by their caloric state. However, ethanol consumption in humans is not thought to be motivated by ethanol's caloric value, as ghrelin has been shown to have no effect on ethanol craving in humans diagnosed with AUD (Kraus et al., 2005). It is therefore likely that ethanol consumption in humans is motivated not by caloric need, but is rather motivated by its reinforcing effects, similar to other drugs of abuse (Spanagel and Weiss, 1999). Given these findings, the ability to generalize the results of data collected in calorically deprived animals to the human condition does not appear prudent, as using these techniques to induce consumption is an inadequate model for AUD.

Future direction: Phasic DA during withdrawal

A future direction of particular interest arising from the present work is understanding whether DA release evoked in response to presentation of ethanol-predictive cues changes when rats are withdrawn from ethanol dependence (Hunter et al., 1974; Schulteis et al., 1996; Weiss et al., 1996). Researchers using *in vivo* microdialysis to measure extracellular DA concentrations in the NAcc during withdrawal from ethanol found that DA signaling was attenuated during withdrawal (Weiss et al., 1996). This result suggests that ethanol seeking and consumption during withdrawal may be motivated by attenuated extracellular levels of DA, as described by the opponent process model of drug-seeking (Solomon and Corbit, 1974; George et al., 2012). Yet, in Chapter 3 of this dissertation (Figure 3.5), I describe potentiated phasic DA signaling during trials in which the rat was more motivated to consume ethanol. My own findings thus corroborate the "incentive salience" theory, which posits that DA has a role in mediating the “wanting” or incentive motivation for reward (Berridge and Robinson, 1998). To resolve the discrepancy between data described herein and the *in vivo* microdialysis literature, recording phasic DA release via FSCV in rats during withdrawal from ethanol in dependent animals would be informative. My work and incentive salience theory would predict a potentiation in phasic DA to accompany the enhanced motivation to consume ethanol under such conditions. Opponent process theory and the supporting *in vivo* microdialysis data would predict an attenuation of DA release. Characterizing and identifying the DA signaling underlying enhanced motivation for ethanol during withdrawal could be of significant value in understanding

motivational mechanisms contributing to relapse in patients suffering from AUDs.

The role of the lateral habenula in learning about the aversive consequences of ethanol

The habenular complex has been strongly implicated in learning about the aversive effects of drugs of abuse, particularly cocaine and nicotine (Fowler et al., 2011; Jhou et al., 2013; Meye et al., 2015). In experiments described in this dissertation, I investigated a role for the habenula in mediating aversion to ethanol. Lesion of the lateral habenula (LHb) was associated with an acceleration in the rate at which rats escalated their intake of ethanol during intermittent ethanol access (IEA) in the home cage relative to a control group, leading to higher levels of consumption after the escalation plateaued. After plateauing, intake levels remained higher in lesioned animals, even after a 42 day interruption of ethanol access. In addition, operant ethanol self-administration was potentiated in lesioned animals. The increases in intake are unlikely to be from differences in ethanol metabolism caused by lesion (Figure 4.1e). Indeed, rats with LHb lesions had elevated blood ethanol concentrations (BECs) both after IEA and operant self-administration. These sustained and consistent differences in intake caused by lesion of the LHb suggest a robust effect of the LHb on ethanol consumption.

The likely mechanism for the LHb to play such a critical role in ethanol consumption is hypothesized to be the contribution of the LHb to learning about the aversive effects of ethanol. Rats with a lesion of the LHb would not learn about the aversive properties of ethanol and, thus, would consume more. One approach

to test this hypothesis is to examine ethanol-induced conditioned taste aversion (CTA) in rats with lesions of the LHb. In such a CTA task, a novel tastant is paired with an aversive injection of ethanol, inducing an aversive association between the novel taste and effects of the drug. Such conditioned aversion thus leads to decreased intake of the tastant. As shown in Chapter 4, rats with a lesion of the LHb recovered their intake of the novel tastant more rapidly than sham-lesioned rats after ethanol-induced CTA. This decreased time to recover in the lesioned rats indicates that the LHb mediates learning about aversion induced by ethanol consumption.

Future direction: Recording from the LHb

The results outlined in Chapter 4 of this dissertation strongly suggest that the LHb mediates learning about the aversive effects of ethanol. Despite this implication, the exact mechanism underlying the role of the LHb in mediating aversion to ethanol remains unclear. At present, it is currently unknown how activity of the LHb encodes aversion to ethanol. Due to the fundamental nature of this question, ongoing studies from the lab are underway address this question. Preliminary results from in *vivo* electrophysiology experiments show that LHb neurons increase their firing after delivery of a tastant that was paired with an aversive ethanol injection (Tandon and Taha, unpublished results). Furthermore, these increases in activity have been found to be predictive of the time to lever press for the associated tastant. While still preliminary, these results are consistent with a model of increased activity of LHb neurons mediating aversion to ethanol.

Future direction: The role of rostromedial tegmental nucleus

Projections from the LHb to the rostromedial tegmental nucleus (RMTg) play an important role in mediating aversion to cocaine (Jhou et al., 2013), but it is currently unknown whether the same pathway mediates aversion to ethanol. The LHb projects to many other regions besides the RMTg, and thus it is possible that aversion to ethanol may be mediated by one of these other efferent pathways A recent report in zebrafish identified the projection of the habenula-to-dorsal raphe connections as playing an important role in learning aversively motivated behavioral responses (Amo et al., 2014). However, preliminary work from the lab has shown that lesion of the RMTg causes an increase in ethanol intake and attenuates learning of an ethanol-CTA (Sheth and Taha, unpublished results). This finding strongly suggests that aversive learning about ethanol requires an intact LHb-to-RMTg pathway. Furthermore, because the majority RMTg efferents project to the ventral tegmental area (VTA) (Jhou et al., 2009b), the role of this pathway in ethanol-conditioned CTA may be mediated by DA signaling.

Discussion of a mechanistic model of how reward and aversion mechanisms contribute to ethanol-seeking behavior

LHb activity may affect phasic DA signaling for ethanol

While not fully established, there is a strong suggestion that the LHb is affecting motivation to consume ethanol by modulating DA release via the LHb-RMTg-VTA pathway. In this dissertation, I have presented data that suggest that phasic DA release in the NAcc may influence motivation for ethanol (Chapter 3).

In addition, I presented data demonstrating the role of the LHb on ethanol consumption (Chapter 4). Putting these results into context with findings implicating the LHb-RMTg-VTA pathway in learning about the aversive effects of cocaine (Friedman et al., 2010; Jhou et al., 2013; Meye et al., 2015), it seems likely the LHb exerts its effects on ethanol-seeking via a similar pathway. In addition, neural activity of the LHb decreases DA neuron activity and decreasing LHb neural activity leads to increased DA release (Christoph et al., 1986; Lecourtier et al., 2008). Furthermore, negative regulation of DA neuron activity by the LHb has been shown to occur in a phasic manner for reward predictive cues (Bromberg-Martin et al., 2010b). Given these findings, there is a strong possibility that the LHb may affect phasic DA release and thereby influence drinking behavior (Figure 5.1).

Phases of ethanol consumption in the proposed model

All of the studies described in this dissertation used an IEA paradigm to induce ethanol consumption. In this paradigm, ethanol consumption progressed through four phases. On the first day of ethanol exposure, ethanol consumption occurred at moderate levels (phase one). The second phase occurred on the second day, at which time most animals exhibited attenuated ethanol intake compared to the first day (phase two). The third phase occurred over the next several weeks during which time consumption slowly escalates (phase three). Finally, the fourth phase occurred when the animal has plateaued at high consumption levels, typically after 1 month of ethanol access (phase four). Interestingly, robust attenuation of consumption in the second phase is predictive

of low levels of ethanol consumption after drinking levels have plateaued ($r = 0.46$, $P<0.05$; Figure 5.2). These results are significant because it suggests that the strength of the initial aversion is related to later drinking behavior. Given the results presented in this dissertation, the correlation is consistent with a model where LHb aversion occurs with initial exposure and this initial aversion is reduced over subsequent exposures to ethanol.

A model for the LHb-RMTg-VTA pathway in mediating ethanol-seeking

Given this evidence, I propose a model for how activity along the LHb-RMTg-VTA pathway influences drinking during the phases outlined above (Figure 5.3). Initial drinking in phase one occurs with little activation of the LHb. Rather, the activity of the LHb is conditioned by aversive effects that occur after cessation of drinking in the first phase. Subsequent exposures to ethanol in phase two (early drinking) then trigger robust activation of the LHb, thereby causing a reduction in DA release upon exposure to ethanol-predictive cues. As decreased DA likely leads to attenuated motivation for ethanol, this would result in the attenuated intake observed in the second phase.

Over many subsequent exposures to ethanol (escalating drinking) in phase three, activation of the LHb may become attenuated, leading to an increase in DA for ethanol. A possible mechanism for this is attenuated activation is from the ventral pallidum (VP), which sends inhibitory projections to the LHb (Hong and Hikosaka, 2013). Neurons of the VP are known to encode expected value, in a

manner similar to LHb activity (Tachibana and Hikosaka, 2012). Ethanol has been shown to decrease levels of GABA in the VP (Kemppainen et al., 2010). Furthermore, administration of GABA-A receptor agonists to the VP decrease ethanol intake, while antagonists increase intake (Kemppainen et al., 2012). These results are consistent with a possible mechanism where inhibition from the VP reduces LHb activity for ethanol-associated cues, possibly during the third phase of drinking.

The slowly diminishing activation of the LHb leads to progression in ethanol-seeking behaviors because there is less suppression of DA release. Lastly, in phase four drinking has plateaued, LHb excitation no longer occurs and LHb activity may actually become inhibited upon ethanol exposure. During this final plateaued phase of consumption, there are likely other mechanisms of aversion act to inhibit further intake. Another region that has been identified in learning CTA is the amygdala (Dunn and Everitt, 1988). The activation of the amygdala with GABA antagonists have been identified in reducing ethanol intake (Hyytiä and Koob, 1995), and may represent an additional mechanism for limiting intake.

Future directions to further support the model

Although the proposed model is consistent with the presented data, there are significant components of the model that have not yet been empirically tested. Such experiments have already begun to address two of the most fundamental of these questions: the activity of the LHb during ethanol-induced aversion and the role of the RMTg in the aversive properties of ethanol. Preliminary data from these

studies are consistent with the proposed model and further implicate the LHb-RMTg-VTA pathway in ethanol aversion (see a *Model for the LHb-RMTg-VTA pathway in mediating ethanol-seeking* above).

Despite this crucial preliminary data, other unsubstantiated components of the model remain and must be addressed by future studies. First, it will be critical to assess the activity of the LHb across the phases of development of voluntary ethanol consumption. While activity of the LHb appears to encode aversion to ethanol, it is unclear if its activity changes with ethanol experience across different phases of ethanol consumption. Likewise, the effects of repeated ethanol consumption on DA release is relatively unknown. The relationship between increased DA and enhanced motivation for ethanol suggests the possibility that the magnitude of phasic DA release in response to ethanol-predictive cues gradually increases with experience, corresponding to the escalation in ethanol consumption in phase three. Furthermore, a gradual reduction in aversion is hypothesized over the same time, as suggested by studies showing that aversive reactions to intraoral infusions of ethanol are attenuated by repeated exposure (Kiefer and Dopp, 1989; Bassareo et al., 2003). While such hedonic reactions, including those related to aversion, have been linked more to the “liking” than the “wanting” pathway, the activity of these pathways have been suggested to be correlated (Berridge, 2000). Given the evidence presented in this dissertation, I hypothesize that LHb is affected by the repeated exposures I show lead to escalated ethanol intake.

It is also possible that attenuated activation of the LHb during phases three

and four (escalating and plateaued intake, respectively) may not occur with repeated exposure, but rather other mechanisms may underlie an increase in phasic DA signaling over time in phase three. For example, a reduction in the activity of GABAergic interneurons of the VTA or direct pharmacological effects of repeated ethanol exposure on DA neurons may contribute (Kohl et al., 1998; Brodie et al., 1999b). Such changes in levels of excitation and inhibition to the VTA may be sufficient to overcome inhibition postulated to be exerted by the RMTg. To address these questions, recordings of LHb activity and DA release must be performed in parallel throughout the escalation period.

Second, it will be critical to establish causal, rather than correlational, evidence for the role of the LHb in the development of voluntary ethanol consumption. The preliminary electrophysiology results, while informative, are only correlational. Likewise, the described lesion studies, while causal, do not specifically implicate the LHb-to-RMTg pathway, but rather only demonstrate that these two regions are involved. The evidence for involvement of this pathway would be strengthened by blocking ethanol aversion by specifically attenuating the activity of LHb neurons projecting to the RMTg. This can be performed by injection of virus expressing the optogenetic construct ArchT into the RMTg. Subsequent delivery of optical stimulation to the LHb will lead to silencing of those neurons that project to the RMTg. Given my model, I would expect the CTA to be abolished during silencing of the LHb-to-RMTg pathway.

Third, it will be fundamental to establish whether LHb activity is entrained after the first exposure to ethanol (*i.e.*, initial drinking). An alternative explanation

is that LHb activation occurs at the onset of drinking, and any drinking that occurs on the first day is driven by other mechanisms. To address this question, the activity of the LHb will have to be recorded during the first and second exposures to ethanol. The initial reduction in drinking appears to play an important role in future drinking behavior given the correlation between the reduction of drinking in phase two and escalated ethanol consumption levels (see Figure 5.2). Therefore, determining whether the LHb is conditioned by the initial aversive effects of ethanol may further our understanding of how mechanisms of aversion may contribute to the high drinking that occurs after the development of AUD.

Finally, I must determine whether activation of the LHb in response to ethanol-predictive cues affects phasic DA release. Phasic reductions in DA have been recorded in response to a cue predictive of foot shock (Oleson et al., 2012). While phasic activation of DA neurons secondary to inhibition of the LHb has been shown by electrophysiology studies (Bromberg-Martin et al., 2010b), no investigations have identified phasic reductions in DA release that are driven by ethanol-related cues. Answering the above questions will be essential to validating the proposed model and determining the precise role of the LHb in ethanol-seeking.

Implications for findings in treatment of AUD

The linking of phasic DA to motivation for ethanol provides a possible therapeutic target for novel therapeutics for AUD. Indeed, a compound that attenuated phasic DA for ethanol may therefore attenuate drinking behavior. A new

compound described as a dopamine stabilizer, called OSU6162, has recently become of interest due to its ability to reduce drinking in rat models and to reduce ethanol-evoked tonic DA release (Steensland et al., 2012). It is currently unknown what, if any, affect it has on phasic DA but it is possible that part of the reduction in ethanol-seeking comes from an attenuation in phasic DA.

The identification of the LHb as a region that mediates aversion to ethanol may also have clinical implications. Deep brain stimulation (DBS) in rats has already been shown to decrease cocaine-seeking in rats (Friedman et al., 2010). My results suggest similar results would be seen during ethanol-seeking. Furthermore, DBS has already been performed in humans in an afferent to the LHb, thereby suggesting the region is viable for this procedure (Sartorius et al., 2010). Although further work is required the safety and efficacy of such a procedure, the work presented suggests a possible treatment avenue.

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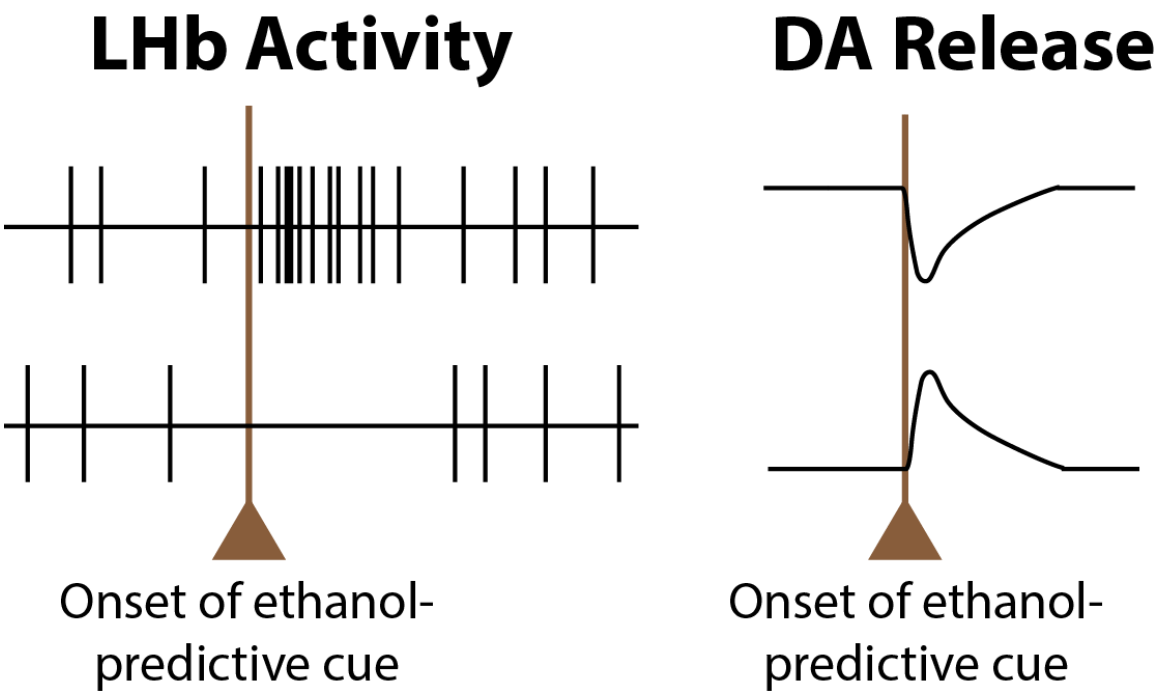

Figure 5.1. A proposed model for how LHb activity may affect DA release

In the model proposed above, phasic increases or decreases in LHb activity have differential effects on DA release.

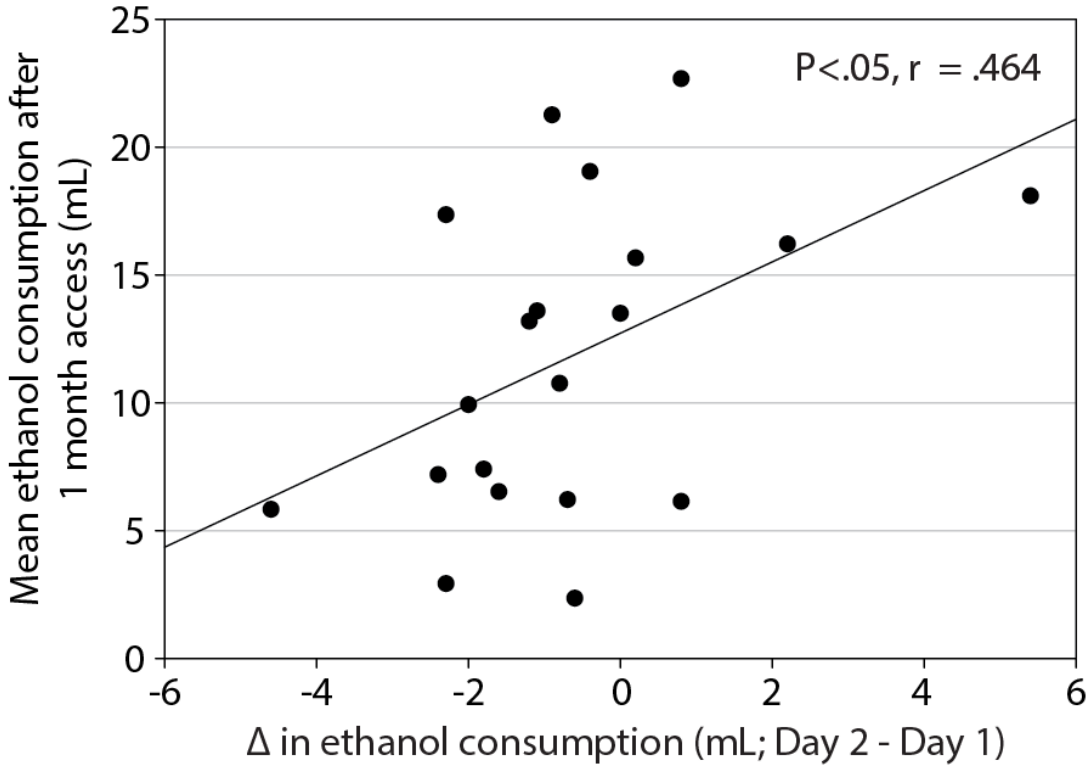

Figure 5.2. First day of consumption predicts future drinking

Change in ethanol consumption between the first two presentations of ethanol is predictive of future ethanol consumption in intact animals (n = 20 rats).

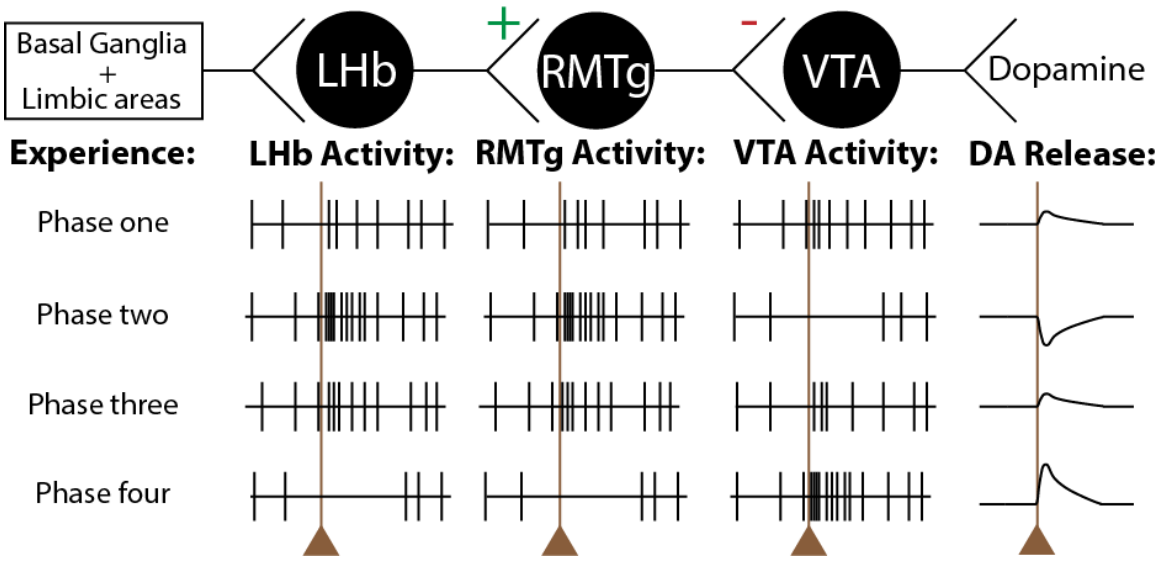

Figure 5.3. A proposed model for how changes in activity may occur in the LHb-RMTg-VTA pathway during escalation of ethanol consumption

Initial exposures to ethanol (phase one) result in little LHb activation (vertical lines) that allows some DA release. Subsequent exposures to ethanol results in robust LHb and RMTg activation (phase two). The robust RMTg activation leads to suppression of VTA activity and an inhibition of DA release. As rats become more experienced with ethanol (phase three), LHb/RMTg activation reduces, leading to increased DA release. Finally, after consumption has plateaued (phase four), activity of the LHb/RMTg is likely suppressed, leading to peak DA release for ethanol.